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# Respirable Particles and Mists in Mouse Pulmonary Infectivity Model. Effect of Chronic or Intermittent Exposure

**IIT Research Inst, Chicago, Ill**

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# Respirable Particles and Mists in Mouse Pulmonary Infectivity Model.

## Effect of Chronic or Intermittent Exposure

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The effects of respirable-sized sulfuric acid mist or mixtures containing acid mist and carbon particles (A-C) on the susceptibility to bacterial and viral respiratory infection were studied in mice and hamsters. Both species showed mortalities upon single 3-hour exposure to 600 mg/m$^3$ but not 400 mg/m$^3$ acid mist. Scanning electron microscopic examination indicated that the most severe changes, including emphysemic-like areas in alveoli, were found after five daily 3-hour exposures to 200 mg/m$^3$ A-C. Significantly increased mortality and decreased bacterial clearance from lungs were also observed in mice challenged with Streptococcus sp.. Significantly increased mortality and pulmonary consolidation, with concomitant decreased survival time, occurred in mice challenged with influenza virus aerosol and exposed to 50 mg/m$^3$ A-C, 3 hr/day, 5 days/week for 4 weeks. Depressed secondary immune responses, as measured by serum antibody levels, were observed in various groups of vaccinated mice exposed to pollutant. The effects of long-term exposure to mixtures of approximately 1.4 mg/m$^3$ sulfuric acid mist and 1.5 mg/m$^3$ carbon particles as well as carbon only were determined. Significant alterations of immunoglobulin concentration, depression of primary antibody response in spleen cells and decreased resistance to respiratory infection as measured by mortality, survival time, and pulmonary consolidation after 20 weeks of exposure were evident.

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# RESPIRABLE PARTICLES AND MISTS IN MOUSE PULMONARY INFECTIVITY MODEL

## Effect of Chronic or Intermittent Exposure

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The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

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The overall objective of this research was to determine if exposure to respirable sized sulfuric acid mist as well as sulfuric acid mist and carbon particle mixtures can alter the resistance of animals to bacterial and viral respiratory infection. The concentration-time relationships for exposure to these pollutants were examined. The parameters measured to define health effects were: mortality, mean survival time, lung edema, lung consolidation, the rate at which viable microbes are cleared from the lungs, histopathologic and scanning electron miscroscopic examination of the lungs, trachea and nasal cavities. In addition, the effects of these exposures on the pulmonary cellular (alveolar macrophages) and mucociliary defense system as well as measuring the immunocompetence of the host was examined in this study.

F. G. Hueter, Ph. D.
Acting Director,
Health Effects Research Laboratory

## ABSTRACT

The effects of respirable-sized sulfuric acid mist or mixtures containing acid mist and carbon particles (A-C) on the susceptibility to bacterial and viral respiratory infection were studied in mice and hamsters. Throughout the short-term exposures, varying concentrations of acid mist were used and combined with 5 mg/m$^3$ carbon particles. Toxicity range-finding studies indicated that hamsters were more resistant than mice, both species showing mortalities upon single 3-hr exposure to 600 mg/m$^3$ but not 400 mg/m$^3$ acid mist. A single 3-hr exposure of hamster tracheal organ culture to mixtures containing 0.8 - 1.4 mg/m$^3$ acid and 1.1 - 1.9 mg/m$^3$ carbon resulted in reduced ciliary activity and damage to the ciliated epithelium. In both *in vivo* and *in vitro* exposure the mixture of the pollutants produced a greater effect than the individual pollutants.

Three combinations of A-C concentrations and durations of exposure resulting in an acid concentration-time (CT) index of 1000 were examined in mice. Scanning electron microscopic examination, which proved to be much more sensitive than histopathologic evaluation, demonstrated that concentration of acid was the most critical factor in producing tissue damage. The most severe changes, including emphysemic-like areas in alveoli, were found after five daily 3-hr exposures to 200 mg/m$^3$ A-C. Significantly increased mortality and decreased bacterial clearance from lungs were also observed in mice challenged with *Streptococcus sp.* Significantly increased mortality and pulmonary consolidation, with concomitant decreased survival time, occurred in mice challenged with influenza virus aerosol and exposed to 50 mg/m$^3$ A-C, 3 hr/day, 5 days/week for 4 weeks. The effects on formation of antibody and on preformed antibody were examined in mice exposed for 3 hr/day, 5 days/week to 100 mg/m$^3$ A-C before or after vaccination with influenza A$_2$/Japan virus. Significantly increased pulmonary consolidation and depressed secondary immune responses, as measured by serum antibody levels, were observed in various groups of vaccinated mice exposed to pollutant compared to vaccinated controls maintained in clean air.

The effects of long-term exposure of 3 hr/day, 5 days/week for 4, 12, or 20 weeks to mixtures of approximately 1.4 mg/m$^3$ sulfuric acid mist and 1.5 mg/m$^3$ carbon particles as well as carbon only were determined in mice. There were significant alterations of immunoglobulin concentration, depression of primary antibody response in spleen cells to antigenic stimulation, and decreased resistance to respiratory infection as measured by mortality, survival time, and pulmonary consolidation after 20 weeks of exposure. Bactericidal capacity of lungs was also reduced in mice exposed to either A-C or to carbon alone, and subtle tissue changes were seen in the respiratory tract upon SEM examination.

These studies were conducted by IIT Research Institute for the Environmental Protection Agency in fulfillment of Contract No. 68-02-1717 from June 3, 1974 to June 2, 1977.

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# LIST OF ABBREVIATIONS

| Abbreviation | Term |
|---|---|
| A-C | acid mist/carbon particle mixture |
| AM | alveolar macrophages |
| BSA | bovine serum albumin |
| CMD | count median diameter |
| CT | concentration x time (index) |
| HBSS | Hanks' balanced salt solution |
| HI | hemagglutination-inhibition |
| Ig | immunoglobulin class |
| $mg/m^3$ | milligram per cubic meter |
| MMD | mass median diameter |
| μ | micron |
| μg | microgram |
| $NO_2$ | nitrogen dioxide |
| PBS | phosphate-buffered saline |
| PFU | plaque-forming unit |
| ppm | parts per million |
| RMSR | relative mean survival rate |
| SD | standard deviation |
| SEM | scanning electron microscopy |
| SN | serum neutralization |
| $SO_2$ | sulfur dioxide |
| SRBC | sheep red blood cells |
| TLV | threshold limit value |

# SECTION 1

# INTRODUCTION

Studies on sulfuric acid mists include animal studies of mortality, pathology, pulmonary function (1,2,3) and combined-effects studies involving challenge with bacterial and viral infectious agents. Such studies show that in addition to the influence of concentration and exposure duration (4,5,6), toxicity of sulfuric acid mists is enhanced by cold (6) and humid conditions (7). This, in part, explains enhanced acid concentrations and toxic effects encountered in urban areas under foggy conditions. Simultaneous exposure to ammonia neutralizes the toxic effects (6,7). Salem and Cullumbine (8) and Boren (9) have shown enhancement of toxicity of certain atmospheric pollutants by kerosene smoke and such carriers as carbon particles. Particle size is also an extremely important factor in determination of the toxicity of an acid mist.

Pulmonary function parameters (10) were used to examine the physiologic response of guinea pigs to low concentrations of sulfuric acid mists of several particle sizes (11,12). It was found that the toxicologic response depends on the particle size as well as the atmospheric concentration since particle size is an important determinant of the amount of toxic substance penetrating the upper respiratory tract, as well as the site of deposition of material reaching the lungs.

Long-term continuous exposure of several species to low concentrations of sulfuric acid mist produced changes in pulmonary function and in the histology of the respiratory tract. Pulmonary function measurements on dogs that received sulfuric acid mist demonstrated loss of functional parenchyma and the development of obstructive pulmonary defects in the conducting airways (13).

Alarie, et al. (14,15) examined the effects of long-term continuous exposure to sulfur dioxide, sulfuric acid mist, fly ash, and their mixtures on histopathology and pulmonary function in monkeys and guinea pigs and found the effects detected from exposures to mixtures could be attributed to the presence of acid mist alone.

Relatively few studies have been done to examine the interaction of exposure to acid mists and defense mechanisms against respiratory infections. Fairchild, et al. (16) observed that exposure of guinea pigs to sulfuric acid mist resulted in greater total respiratory deposition of radiolabeled streptococcus aerosol than control animals and a proximal shift in the regional pattern of deposition to the nasopharynx. Inhalation of sulfuric acid mist aerosol by mice before or after exposure to radioactive aerosol also impaired the mucociliary defense mechanism of the respiratory tract (17).

The overall objective of this project was to determine if exposure to respirable-sized sulfuric acid mist as well as sulfuric acid mist and carbon particle mixtures (A-C) alter the resistance of a laboratory animal host to bacterial and viral respiratory infection.

During the initial phase of the program, the methodologies required for exposure of animals to the pollutants, including the generation of sulfuric acid mist and particulate aerosols and monitoring of the aerosols, were established. Preliminary range-finding experiments were conducted to determine the toxicity of the pollutants resulting from a single 3- or 6-hr exposure. This information was then utilized to design combined-effects studies employing a single acute pollutant exposure.

A series of experiments was then initiated to determine the effects of multiple exposures to these pollutants on the host's resistance to infection. Mice were exposed 5, 10, and 20 times to 5 mg/m$^3$ carbon only (3 hr/day, 5 days/week) immediately before or after challenge with influenza virus. We then determined the lowest concentration of A-C mixtures and number of repeated exposures to the mixture that produced increased mortality in conjunction with either a bacterial or viral challenge. The concentration-time (CT) relationship for exposure to A-C and infectious challenge was examined. The parameters measured to define the health effects were: mortality, mean survival time, lung edema, lung consolidation, the rate at which viable bacteria are cleared from the lungs of mice, histopathologic examination of appropriate tissues, and scanning electron microscopic observation of lung, trachea, and nasal cavities. In limited experiments, the effects of exposures on the pulmonary cellular defense system of mice were determined. These included the examination of total and differential cell counts, viability, cell surface morphology and *in vitro* phagocytic function of alveolar macrophages lavaged from lungs, as well as studies of the *in vivo* bactericidal capacity in the lungs. These studies were summarized in Annual IITRI Report L6080-4 (1975) and IITRI Report L6080-8 (1976) and the data reported in the open literature (18,19).

Studies of the effect of exposure to A-C mixtures on vaccine-induced immunity to influenza virus were completed. Mortality, mean survival time, lung consolidation, serum neutralization (SN) and hemagglutination-inhibition (HI) antibody titers were measured. In addition, preliminary studies were initiated to determine the effects of a single 3-hr exposure to 1.4 ± 0.4 mg/m$^3$ sulfuric acid mist and/or 1.5 ± 0.4 mg/m$^3$ carbon on tracheal epithelium in organ culture. Changes in ciliary activity were determined and alterations in tracheal epithelium following exposure to A-C were examined by conventional and scanning electron microscopic techniques.

The effects of long-term (4,12 and 20 week) exposures (3 hr/day, 5 days/week) to 1.4 ± 0.4 SD $mg/m^3$ acid mist and 1.5 ± 0.4 $mg/m^3$ carbon as well as carbon only were examined in normal mice and in combined-effects studies with influenza virus challenge. The effects of the exposure on normal (uninfected) mice were examined using the following parameters: hematology, blood chemistry, immunoglobulin concentration, Jerne plaque assay for number of plaque-forming cells in the spleen, bactericidal assay in lungs, examination of the respiratory tract by SEM and conventional microscopy, and body weight and temperature measurements. Parameters for examining the effects of exposure on the response to challenge with infectious influenza virus included mortality, mean survival time, and lung consolidation.

# SECTION 2

# CONCLUSIONS

Data were accumulated on single, 3-hr exposures to aerosols of sulfuric acid mists and A-C. Mortalities were found at 600 mg/m$^3$ acid mist, whereas the animals tolerated 400 mg/m$^3$ and below. Hamsters were more resistant to acid mist per se than mice. The effects of single and multiple 3-hr inhalation exposures to varying concentrations of sulfuric acid mist in combination with a constant concentration of 5 mg/m$^3$ of carbon particles were examined. As was true with acid mist alone, mice were more sensitive to A-C than hamsters. Only minimal mortality occurred among CF$_1$ mice after a single 3-hr exposure to 300 mg/m$^3$ or five daily 3-hr exposures to 200 mg/m$^3$ A-C.

Three combinations of exposure duration and varying acid concentration combined with 5 mg/m$^3$ of carbon particles resulting in the same concentration-exposure time index (CT) were examined: 5 daily 3-hr exposures to 200 mg/m$^3$, 10 daily exposures to 100 mg/m$^3$, and 20 daily exposures to 50 mg/m$^3$ A-C. Scanning electron microscopic (SEM) examination of nasal cavities, trachea, and lungs showed that the initial concentration of A-C is a more critical factor in tissue damage than the total number of exposures. Exposure to acid mist alone caused damage near the top and middle of the trachea, while the lower portion of the trachea was relatively free of damage and the lungs were normal. Addition of the 5 mg/m$^3$ carbon particulates to the acid mist extended the damage to the lower trachea and the bronchus of the lungs. The most severe changes, including emphysemic-like areas in the alveoli, were found in mice following five daily 3-hr exposures to 200 mg/m$^3$ A-C. Histopathologic examination of these tissues did not disclose any marked changes, indicating the sensitivity of SEM in detecting tissue damage.

Infectivity studies conducted with various concentrations of acid in conjunction with a constant concentration of 5 mg/m$^3$ of carbon particles demonstrated the following.

- Increased mortalities occurred in hamsters after a single 6-hr exposure to 300 mg/m$^3$ A-C 24 hr or 48 hr after intranasal challenge with influenza virus.

- Five daily 3-hr exposures to 200 mg/m$^3$ A-C immediately before or after challenge with a viral or bacterial agent significantly increased mortality of mice. A concurrent marked decrease in clearance rate of inhaled bacteria from the lungs was observed.

- Five as well as ten daily 3-hr exposures 5 days/week to 100 mg/m$^3$ A-C immediately after challenge with a bacterial agent also resulted in markedly increased mortality and decreased survival time. Significantly increased mortality and decreased survival time were seen in mice exposed daily for 3 hr for 5 days to 100 mg/m$^3$ A-C 24 hr before or after challenge with influenza virus.

- Twenty 3-hr exposures, 5 days/week, to 50 mg/m$^3$ A-C followed immediately by challenge with influenza virus resulted in significantly increased mortality and decreased survival time in experimental mice compared to controls.

Studies of the cellular defense system indicated a decreased capacity to clear bacteria in lungs of mice infected immediately after a single 3-hr exposure to 100 mg/m$^3$ acid and 5 mg/m$^3$ carbon mists. Parallel extensive structural changes were seen upon scanning electron microscopic examination of alveolar macrophages lavaged from their lungs immediately after A-C exposure.

Studies of the effects of multiple exposures (3 hr/day, 5 days/week) to 5 mg/m$^3$ of carbon aerosols showed that 5 or 10 exposures either immediately before or after challenge with airborne influenza virus caused no enhancement of mortality or alteration of mean survival time of male CD$_1$ mice. However, 20 daily exposures immediately before virus challenge resulted in significant increase in mortality and pulmonary consolidation. Upon SEM examination of the respiratory tract, slightly increased sloughing of squamous cells in the external nares was seen, whereas the remaining respiratory epithelium appeared normal.

The effects of inhalation of A-C on formation of antibody and on preformed antibody were examined in mice exposed for 3 hr/day, 5 days/week to 100 mg/m$^3$ A-C before or after vaccination with influenza $A_2$/Japan virus. At various time intervals after the vaccination, the mice were challenged with homologous influenza virus and mortality, survival rate, pulmonary consolidation and immune response were measured. In terms of mortality and survival time, the vaccine protected A-C and air-exposed animals equally well. However, significantly increased pulmonary consolidation and depressed secondary immune responses, as measured by HI and SN antibody levels, occurred in many groups of vaccinated mice exposed to pollutant compared to vaccinated controls maintained in clean air. In general, there was little effect of exposure to A-C on preformed antibody in studies in which A-C exposure occurred immediately after the infectious challenge which followed vaccination at various intervals. In studies in which A-C exposure occurred immediately before or after vaccination, pulmonary consolidation scores measured after subsequent infectious challenge were generally significantly higher in the vaccinated mice which had been exposed to A-C. The primary SN antibody response was significantly increased when the interval between vaccination and infectious challenge was 8 weeks. Secondary HI and SN antibody responses were generally depressed when challenge occurred 2, 8, or 16 weeks after vaccination.

Examination of the hamster tracheal epithelium in organ culture showed that exposure to A-C reduced ciliary activity with damage to the ciliated epithelium immediately after a single 3-hr exposure to 0.8 to 1.4 mg/m$^3$ acid and 1.1 to 1.9 mg/m$^3$ carbon. In both *in vivo* and *in vitro* exposure the acid-carbon mixture produced a greater effect than the two pollutants singly. Recovery from the acid-carbon mixture was similar to explants exposed to acid alone. Carbon-exposed explants recovered to an activity index similar to the controls. There was a clear correlation between initial damage to tracheal cells in organ culture and their recovery, and the ability to reproduce these effects in hamsters.

Long-term exposures of mice 3 hr/day, 5 days/week for 4, 12 or 20 weeks to 1.4 ± 0.4 (SD) mg/m$^3$ sulfuric acid mist and 1.5 ± 0.4 mg/m$^3$ carbon aerosol as well as carbon only demonstrated the following:

- Subtle changes were detected by SEM examination of the respiratory tract. After 12 and 20 weeks exposure to either carbon or A-C, increased sloughing of squamous cells in the nasal cavity and areas of thickened alveoli in the lungs were observed. The changes were more widespread in animals exposed to A-C.

- Mice exposed to A-C showed markedly slower weight gain than air controls during the period of rapid growth in the first 8 weeks of exposure to the pollutant.

- Serum immunoglobulin concentrations varied in response to exposure to pollutants, with the changes more marked in mice exposed to A-C than to carbon. Concentrations of IgA did not change until 20 weeks exposure when they were slightly depressed. Levels of $IgG_1$ were depressed during the first 4 weeks of the A-C exposure. Concentrations of $IgG_{2a}$ slightly increased initially in the A-C exposure group, then depressed from 4 to 12 weeks of exposure. $IgG_{2b}$ levels were elevated initially and again after 20 weeks of exposure to both carbon and A-C. Concentration of IgM was elevated after one week of exposure to A-C but depressed throughout the remainder of the 20 week exposure.

- Primary antibody response of spleen cells derived from mice exposed to A-C or carbon assayed by the Jerne plaque technique followed the pattern typical of other stress exposures. The response was stimulated after 4 weeks of exposure, did not differ from the controls after 12 weeks exposure, and a marked depression of the response, especially in mice exposed to A-C, was observed after 20 weeks exposure.

- Bactericidal capacity in the lungs of mice exposed for 4 or 12 weeks to carbon or A-C was significantly reduced compared to air controls. Loading of the macrophages with carbon was apparently the most significant factor in the decrease. A slight reduction in bactericidal activity remained after 20 weeks of exposure.

- Marked increases in mortality and pulmonary consolidation and decreased survival time were seen in mice exposed to A-C for 20 weeks and challenged with influenza $A_2$/Taiwan virus.

The studies indicated that the effects of long-term exposure to mixtures of concentrations of $1.4 \pm 0.4$ mg/m$^3$ sulfuric acid and $1.5 \pm 0.4$ mg/m$^3$ carbon (0.4 μMMD) as well as carbon only (0.3 μMMD) have been detected using sensitive parameters. The immunologic state of the animal was examined directly in response to specific antigens by the primary response of spleen cells, and indirectly by infectivity studies. A quantitative measure of the effects on the immune system without antigenic stimulation was obtained by determination of serum immunoglobulin concentrations. These parameters have shown significant alterations of immunoglobulin titer, depression of primary antibody response in spleen cells to antigenic stimulation, and decreased resistance to respiratory infection as measured by mortality, survival time, and pulmonary consolidation after 20 weeks of exposure to A-C. In addition, bactericidal capacity of lungs was reduced in mice exposed to either A-C or to carbon alone, and subtle morphological changes in the respiratory tract were detected by SEM.

The alterations of the pulmonary defense system suggest that prolonged exposure to low concentrations of sulfuric acid and carbon particle mixtures reduce the ability of mice to resist the secondary stress of respiratory infection.

# SECTION 3

# RECOMMENDATIONS

To further explore the effects of exposure to low concentrations of sulfuric acid mists on the defense mechanisms of a host, research efforts should be extended to life-time studies on an animal host. Cellular and humoral immunity (B and T cell stimulation), viability and enzyme activity of alveolar macrophages, examination of effects on interferon production and lung clearance mechanisms should be examined in addition to the parameters used in the present study. The effects of the exposure stress on the activation of latent infections should also be examined. In terms of the present model system, regular serologic monitoring of animals in long-term exposure studies is recommended in order that such periodic measurements will detect effects of the exposure stress on the activation of unexpected latent bacterial and viral infections.

# SECTION 4

# MATERIALS AND METHODS

## GENERATION OF ACID MIST AND PARTICULATE AEROSOLS

The apparatus used for generation of the sulfuric acid mist (96% purity, Baker Co., Phillipsburg, NJ) and carbon particle (Sterling MT CT-6729, carbon black 99.5% purity, Cabot Corp. Boston, MA) aerosol is shown in Figure 1. Dry filtered compressed air was regulated by a low pressure regulator. A portion of the air, controlled by valve $V_3$ and monitored by gauge $P_2$, was bubbled through distilled water maintained at room temperature while another portion of the air (controlled by valve $V_2$ and monitored by the flowmeter) mixed with the air stream from the bubbler to provide a 60% RH and a flow rate of 100 liters/min. The third stream of air (controlled by valve $V_1$ and monitored by $P_1$) passed through a DeVilbiss No. 40 nebulizer containing 40% sulfuric acid solution (or a 0.4% sulfuric acid solution for the 1-2 mg/m$^3$ acid studies). The aerosol produced by the nebulizer was diluted with the 60% RH air in a flask and passed into the animal exposure chamber.

To prevent changes in particle size due to evaporation and condensation the humidity in the chamber was maintained fairly constant. Since the size change is most significant when humidity is below 20% or above 80% RH, a humidity of 60% RH was chosen for these experiments.

The carbon aerosol was generated by a Wright dust feeder. A slowly rotating scraper blade continuously released a small quanity of carbon powder. The powder carried by the air stream was impacted on a plate to break up the agglomerates and disperse the particles uniformly in the air. In the short term, multiple-exposure experiments, a mean carbon particle size of 0.3 μm and a concentration of 5 mg/m$^3$ were used.

The A-C aerosol was produced by mixing the sulfuric acid mist and the carbon aerosol stream and passing the mixture through a tube maintained at 310°C. The sulfuric acid vaporized in this tube and, as the mixture cooled in the section following the heated tube, the acid vapor condensed on the solid carbon nuclei. The resulting aerosol was then diluted with the wet and dry air in the three-neck flask to adjust the humidity to 60% RH. The aerosol was then passed into the animal exposure chamber.

The chamber used for exposure of animals to the pollutants is shown in Figure 2. The acid mist was introduced near the bottom at one end of the chamber and exhausted from the top diagonally across from the inlet.

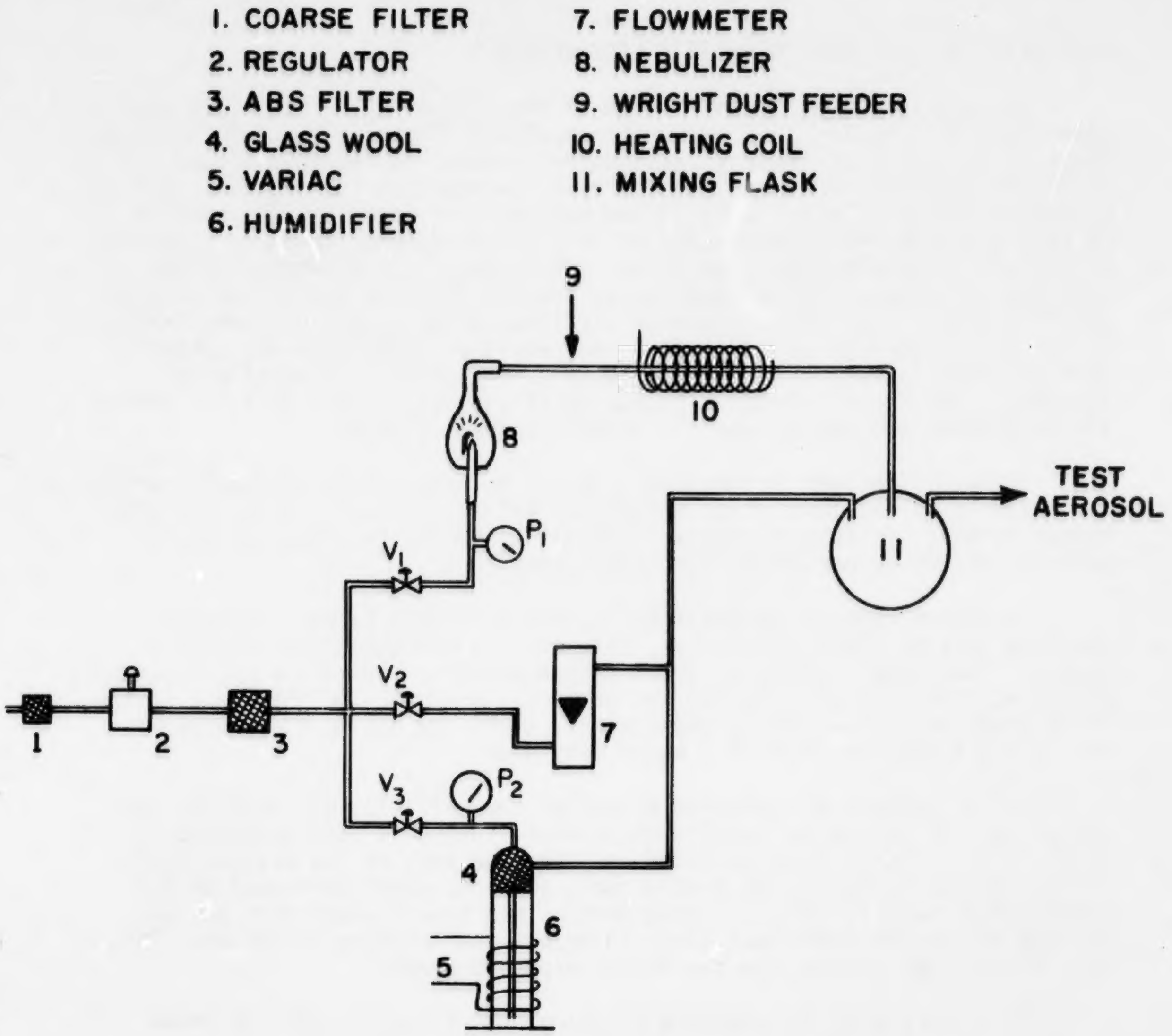
1. COARSE FILTER
2. REGULATOR
3. ABS FILTER
4. GLASS WOOL
5. VARIAC
6. HUMIDIFIER
7. FLOWMETER
8. NEBULIZER
9. WRIGHT DUST FEEDER
10. HEATING COIL
11. MIXING FLASK
9
10
8
TEST AEROSOL
11
P1
V1
V2
7
1
2
3
V3
P2
4
6
5

Figure 1. Sulfuric acid aerosol generator.

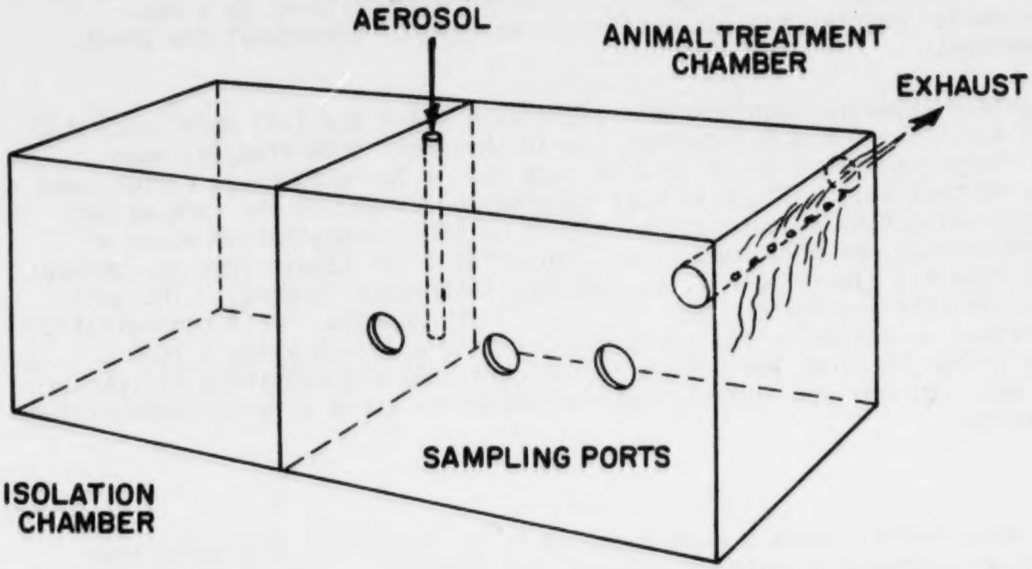
$H_2\ SO_4$
AEROSOL
ANIMAL TREATMENT CHAMBER
EXHAUST
SAMPLING PORTS
ISOLATION CHAMBER

Figure 2. Animal exposure chamber for acid aerosols.

For particle size analysis of all concentrations of acid mists produced by hot generation and for acid coated carbon aerosols with mean particle diameters of 0.12 μm and 0.4 μm, respectively, the aerosol in the chamber was sampled from Port No. 2 using an electric mobility analyzer. The sampling system used for the quantitative assay consisted of two Greenberg impingers in series, each containing 50 ml of isopropanol-water mixture (80:20 v/v). The sample was collected for a known period of time (at least 10 min) at a flow rate of 1 liter/min held constant by a stainless steel critical orifice. The contents of the impingers were transferred to a 200-ml volumetric flask and titrated with 0.001 N barium perchlorate using thorin as an indicator. The acid concentrations used in the experiments ranged from 50 to 200 mg/m$^3$ and were obtained by varying the pressure of the air passing through the nebulizer. The carbon concentration was monitored by a condensation nuclei counter and was maintained at 5 mg/m$^3$ throughout the short-term experiments.

Long-term exposure mean concentrations were 1.4 ± 0.4 (SD) mg/m$^3$ sulfuric acid mist and 1.5 ± 0.4 mg/m$^3$ carbon. As in the short term studies, mean particle diameters were 0.12 μm, 0.3 μm, and 0.4 μm for acid only, carbon, and A-C mists respectively. The acid mist generation system was the same as outlined above using 0.4% acid solution instead of 40%. Quantitative assay of acid concentration was performed by pulling a 200 liter sample from the chamber through a type A-E glass fiber filter (Gelman Instrument Company). The acid was dissolved from the filter into 15 ml of 5% isopropanol. Acid concentration was determined by measuring the resistance of this solution using a 1650-B impedance bridge (General Radio Company, Concord, MA) and comparing to standard curve values. Carbon concentration was monitored as above using a condensation nuclei counter.

## ANIMALS

The experimental animals used included 3- to 6-week-old COBS mice from Charles River Laboratory (male CD$_1$ strain) and ARS/Sprague-Dawley (male and female ARS$_2$-CF$_1$ strain), conventional 3- to 6-week-old BDF$_1$ strain mice from Murphy Breeding Labs, and 12- to 14-week-old male Cr:RGH (SYR) Syrian golden hamsters obtained from ARS/Sprague-Dawley. The animals were quarantined for 2 weeks and were provided with food and water *ad libitum* throughout the experiments. For exposure to pollutants and airborne infectious agents the animals were placed into specially constructed stainless steel wire cages, keeping each animal in a separate compartment. In a few selected experiments, two to five mice were groups per compartment for exposure to pollutant only.

## INFECTIOUS AGENTS

Influenza virus was prepared by rapid passage through mice or hamsters and identified by a NIH reference reagent antiserum. Influenza A$_2$/Taiwan/64 and A$_2$/Japan/170 viruses were used for mice and A/PR/8 for hamsters. Within 24 to 48 hr after intranasal inoculation, lungs were removed aseptically and a 20% suspension was prepared in phosphate-buffered saline (PBS). For intranasal inoculation dilutions were made in PBS; for aerosolization the diluent was PBS containing 0.2% bovine serum albumin. For the vaccine study A$_2$/Japan/170 influenza virus was prepared by rapid passage through mice followed by growth in 10-day-old embryonated chicken eggs and identification by NIH reference reagent antiserum.

*Streptococcus* sp., Lancefield's group C, was grown in Todd Hewitt broth for 18 hr at 37°C. Suspensions of the bacteria were prepared in 0.1% peptone water for aerosolization.

*Klebsiella pneumoniae,* type 1, passaged in mice and isolated from the heart, was grown in trypticase soy broth for 18 hr at 37°C. Suspensions of the bacteria were prepared in 0.1% peptone water for aerosolization.

## VACCINE

Influenza $A_2$/Japan/170/62 virus vaccine (Lot 2JG63, Eli Lilly and Company, Indianapolis, IN) was used. This commercial preparation had been zonal centrifuged and contained 3696 CCA units per ml.

## INFECTIOUS CHALLENGE

The experimental animals were infected by exposure to an aerosol or by intranasal inoculation of the agent. Infectious aerosol challenge was conducted in a 350-liter plastic aerosol chamber (60x60x95 cm) installed within a microbiological safety cabinet. A continuous flow DeVilbiss atomizer (Model 84) was used to disseminate the infectious agent with particles of 1 to 5 μm MMD by means of filtered compressed air. For the challenge, mice were placed in the aerosol chamber and exposed for 5 to 10 min to the airborne infectious agent. Control mice were exposed to the sterile diluent for a comparable interval. After challenge, the animals were removed from the chamber and held for 14 days in an isolated clean air animal room.

## PULMONARY CONSOLIDATION

Lung consolidation in mice was scored on a scale ranging from 0 to 5.

0 = no consolidation
1 = 1 to 25% consolidation
2 = 26 to 50%
3 = 51 to 75%
4 = 76 to 100%

A value of 5 was assigned to mice that died during the experiment (20).

## RELATIVE MEAN SURVIVAL RATE (RMSR)

The RMSR was calculated according to the equation:

$$\text{RMSR} = \frac{\Sigma(AB)+(dL)}{n}$$

where A is the last day on which any individual mouse is alive; B is the number of mice surviving A days; d is the last day of observation; L is the number of mice alive on the day d; and n is the original number of mice in the experimental group (21).

## LUNG CLEARANCE

Groups of four or five mice were killed immediately after challenge with *Streptococcus* sp. and at hourly intervals for up to 6 hr and at 24, 48 and 72 hr. Each lung was aseptically removed, weighed and homogenized in 1.8 ml of sterile peptone water. The homogenates were diluted in 0.1% peptone water and plated in duplicate on blood agar base media (BBL) with 5 to 10% defibrinated sheep blood. The number of viable *Streptococcus* per gram wet weight of lung determined at 0 hr was considered 100% recovery. The microorganisms in lungs at hourly intervals were assayed by the same procedure and the number of viable microorganisms/gram wet weight of lung was compared to the 0-hr value and plotted on semilogarithmic paper.

The rate of bacterial clearance was calculated and expressed as the $t_{1/2}$ value representing the time required for 50% of the original bacterial population to be cleared from the lungs. The $t_{1/2}$ was determined on a semilogarithmic regression after converting the percent recovery to $\log_{10}$ values.

## SEROLOGICAL METHODS

HI antibody tests were performed in duplicate by the microtiter method in disposable V-plates. In all tests, 1% chicken red-blood cells and four hemagglutinating units of egg-adapted influenza $A_2$/Japan/170 virus were used. Type $A_2$ influenza (Japan/170/62) antiserum, received from the National Institutes of Health, served as the positive control.

SN tests were performed in 10-day-old embryonated chicken eggs. Serially diluted serum samples were mixed with equal volumes of 10 to 320 $LD_{50}$ of influenza virus and incubated for 1 hr at 4°C. The mixtures were then inoculated into the allantoic cavity and the eggs incubated for 48 hr. The hemagglutination test was then performed on the harvested fluids by using 0.5% chick red-blood cells. The SN titers were calculated by the Reed-Muench method (22).

## HEMATOLOGY TESTS

### Erythrocyte and Leukocyte

A Coulter Electronic Particle Counter with 100 aperature was used. Each blood sample was counted in duplicate. Reference blood samples (Coulter Electronics, Inc) were counted for standardization (23).

### Hematocrit

Packed cell volume was determined in capillary tubes using a microcapillary head centrifuge (International Equipment Company, Model MB).

### Hemoglobin

Hemoglobin was measured as cyanomethemoglobin, with a reference solution as standard (24).

### Differential Leukocyte Count

Wright's stain was used to stain the leukocytes for examination.

### Reticulocyte Count

These cells were counted by the new methylene blue N method (25).

### Platelet Count

Platelets were counted visually in a hemocytometer with a phase microscope.

## CLINICAL CHEMISTRY TESTS

The following tests were performed on a centrifugal analyzer (CentrifiChem, Union Carbide, Tarrytown, NY) using microassays. Tests were run the day the blood was collected, except in a few instances where technical difficulties required storage of sera at -20°C. Previous work had shown that such storage had no significant adverse effect.

### Alkaline Phosphatase

A modified Bessey-Lowry-Brock technique utilizing p-nitrophenyl phosphate as substrate for AP was used (26).

### Lactic Dehydrogenase-L

The microassay developed by Wacker, *et al*. (27) was used.

### $\alpha$Hydroxybutyrate Dehydrogenase

The microassay developed by Rosalki, *et al*. (28) was used.

### Isocitric Dehydrogenase

The microassay developed by Wolfson, *et al*. (29) was used.

### Lactic Dehydrogenase Isoenzymes

A Beckman Microzone Electrophoresis system was adapted from the method developed by Dade Division (Amer. Hosp. Supply Div.).

## SERUM IMMUNOGLOBULIN CONCENTRATION

Quantitative radial immunodiffusion plates for mouse immunoglobulins IgA, $IgG_1$, $IgG_{2a}$,$IgG_{2b}$ and IgM were procured from Meloy Laboratories, Inc., Springfield, Virginia. Reference standards obtained from pooled sera of normal mice were assayed daily, in duplicate, to provide quality control. Mouse immunoglobulin standards (Meloy Laboratories) were assayed concurrently to quantitate the experimental samples. Duplicate serum samples were placed in preformed wells, the plates incubated at 22°C for 18 hr, and the radial diffusion diameters measured using a Bausch & Lomb 7x lens. Duplicate radial diffusion diameters were recorded for each of the 6 to 9 serum samples.

## JERNE PLAQUE ASSAY

The plaque assay i.e., hemolysis-in-gel first described by Jerne, et al. (30) and modified by Kaliss (31) was used to determine humoral antibody response to sheep red blood cells (SRBC). Six mice from each exposure group were injected intraperitoneally with 0.5 ml of a 2% suspension of washed SRBC in 0.85% saline. After 4 days the spleens were removed and a single-cell suspension prepared from each.

For the plaque assay, 0.6 ml of 1.5% Seaplaque agarose (Marine Colloids) in Hanks' balanced salt solution (HBSS), 0.3 ml of 2% SRBC in 0.85% saline, and 0.3 ml of the appropriate spleen cell suspension were added in sequence to plastic tubes (17 x 100 mm, Falcon) maintained at 42°C. The contents were mixed and poured into petri plates containing a preformed agarose layer (3 ml of Seakem agarose, Marine Colloids, Rockland, Maine at a 1.5% solution in HBSS in 60 x 15 mm petri dishes). Each spleen cell suspension was plated in triplicate. The plates were gelled at 24°C for 10 min and incubated for 1 hr at 37°C in a 5% $CO_2$ incubator. One milliliter of guinea pig complement (Microbiological Associates, Bethesda, MD) at a 20% solution in HBSS was carefully added to each plate and incubated 60 min at 37°C. The plates were stored overnight at 4°C and plaques were counted the following day. Prior work had shown that such storage produced no alterations.

## ALVEOLAR MACROPHAGES

Alveolar macrophages obtained from mice by tracheobronchial lavage were examined for total and differential cell count and viability by conventional methods.

## GROWING RADIOLABELED BACTERIA

Cultures of $^{35}$S-labeled *K. pneumoniae* were grown in Anderson's medium by an adaptation of the method of Berlin and Rylander (32) used for labeling of *E. coli*. In the medium composed of 0.1 g $MgSO_4$, 3.0 g $Na_2HPO_4$, 5.0 g NaCl, 1.0 g $NH_4Cl$ and 4.0 g glucose per 1000 ml, the $MgSO_4$ was replaced by $MgCl_2$ and the sulfate requirement of the bacteria was then provided by addition of $^{35}$S-labeled $Na_2SO_4$. For each experiment, a culture of *K. pneumoniae* was first grown in conventional Anderson's medium for 24 hr from a bacterial stock maintained in trypticase soy broth. The concentration of this bacterial suspension was then adjusted to $10^8$/ml after counting in a Petroff-Hauser chamber, and a 0.1 ml aliquot was used to inoculate 10 ml of Anderson's medium containing ≈180 µCi of $^{35}S\text{-}Na_2SO_4$ (specific activity $\geq$100 mCi/mM). After 16 hr of incubation, the $^{35}$S-labeled *K. pneumoniae* was harvested and the bacteria were washed and centrifuged at 12,000 xg 10 times for removal of unattached radiolabel. All bacterial counts were first determined by dark field microscopy in a Petroff-Hauser bacterial counting chamber and subsequently also by culture plate technique.

## BACTERICIDAL ACTIVITY

Intrapulmonary bacterial inactivation was determined in the lungs of individual animals by the method of Green and Goldstein (33). Mice are exposed to radiolabeled live bacteria and the ratio of the viable bacterial count to the radioactive count in each animal's lung provides a measure of bacteria which are destroyed by the lung at a given time after infection.

After exposure to A-C mist, groups of mice were infected by intranasal inoculation with $^{35}$S-labeled *K. pneumoniae*. Four hr after infection they were killed, and their lungs were aseptically removed and homogenized. From the radioactive counts made on aliquots of the homogenates and the radioactive labeling ratio determined for each bacterial culture, the total number of bacteria deposited in each lung could be calculated immediately. On the basis of this information, another aliquot of each lung homogenate was appropriately diluted for determination by culture-plate technique of the bacteria that remained live in the lungs.

## SCANNING ELECTRON MICROSCOPY AND HISTOPATHOLOGY

Nasal cavities, trachea, and lungs were taken for SEM examination from mice killed immediately after exposure to air, carbon, and A-C mist. The animals were anesthetized by an intraperitoneal injection of pentobarbital, and the abdomen was opened on the midline, permitting access to the ventral aorta. The aorta was severed, the animal exsanguinated, the chest opened, and the lungs and trachea were removed *in toto*. The trachea was cannulated to the level of the first cartilaginous ring and the lungs expanded with Karnovsky's paraformaldehyde-glutaraldehyde phosphate-buffered fixative (34) at 20 cm water pressure. Perfusion continued for at least 2 hr with the lungs completely immersed in fixative. Upon completion of airway perfusion, the trachea was ligated and the lungs floated in fixative.

After cannulation of the trachea, the head was removed, the skin was retracted and the lower jaw removed by sectioning through the ramus. The cranium was cut off immediately posterior to the orbits, leaving only the nasal cavity. The nasal bones were reflected utilizing small forceps and the cavity sectioned in half by inserting a razor blade on one side of the median septum and severing the hard palate.

The trachea and main stem bronchi were isolated from the lungs. The lungs were sectioned with a razor blade so as to reveal the bronchus and alveoli of each lobe. All tissues were washed in distilled water and dehydrated with increasing concentrations of alcohol. Pentyl acetate was then substituted for the alcohol and the tissues dried by the critical point method in carbon dioxide. The dried trachea was sectioned longitudinally, and all tissues were cemented to stubs, gold-coated in a Denton vacuum evaporator equipped with a rotating turntable, and examined in a Kent-Cambridge Mark II Stereoscan scanning electron microscope at 20 kV.

Samples of lung, trachea and nasal cavities were also processed for light microscopy by conventional histologic methods. Sections were cut at 5 μm and stained with hematoxylin and eosin.

## PREPARATION OF TRACHEAL RINGS AND CULTIVATION METHOD

Four-week-old Syrian Golden Hamsters were killed with $CO_2$ and immediately opened from the middle of the sternum to the larynx. The trachea was exposed and the surrounding membranes aseptically teased away with fine forceps. The trachea was gently lifted,and a forceps was slid underneath to free it from the esophagus and to facilitate cleaning. After removing remaining membrane, the trachea was excised, cutting just above the larynx and just above the bifurcation. The trachea was briefly rinsed in HBSS and cleaned of remaining membrane. The larynx-end of the trachea was grasped with a sterile curved hemostat which was laid against the edge of the dish at an acute angle with the trachea held over the dish. Each trachea was opened from the larynx to the corina along the membranous dorsal wall. After grasping the loose end with sterile forceps, the trachea was gently stretched to slightly separate the white cartilage rings. With a sterile scapel the trachea was cut into rings approximately 1 mm thick. Approximately 10 to 12 tracheal rings were obtained from each animal.

Tracheal rings were washed in Eagle's basal medium and placed on a section cross-hatched with a scapel blade in 35 x 10 mm unwettable plastic petri dishes containing 0.7 ml of HEPES buffered CMRL 1066 medium supplemented with 0.2% bovine serum albumin, penicillin (250 U/ml), and streptomycin (250 μg/ml). The rings were incubated for 24 hr at 37°C in 5% $CO_2$ atmosphere and 90% relative humidity. This initial incubation period allows the rings to adjust to the external environment and ensures that normal beating frequency is restored before the test substance is applied.

Immediately following removal from the hamster, all tracheal rings were examined in inverted Nikon microscope at 200X for normal ciliary activity or evidence of cytopathology. The vigor of ciliary activity at the periphery of the explants was determined using an electronic strobescope (General Radio, Type 1531-AB) as the light source. A Sage air curtain (Sage Instruments, Model 279) was placed adjacent to the microscope stage to maintain the temperature at 37°C while determining the ciliary beat frequency. When the flash rate of the strobescope is set to the same speed as the ciliary beat, ciliary movement appears to stop. Ciliary beat frequency of ring explants was measured at four separate areas on the lumen and their average was recorded as the initial beats per minute. The ciliary activity of whole trachea explants was observed by focusing on the mucosa through the explant. After the initial 24 hr incubation period, the baseline cilia beating frequency for each explant was determined. Cilia beating frequency was determined every 24 hr for 3 days in the A-C mixture studies.

For light microscopy, ring explants were washed in Hank's salt solution to remove mucus, fixed in 10% neutral-buffered formaldehyde solution, dehydrated and infiltrated. Paraffin-embedded tissue was cut into 6-μm thick sections and stained with hematoxylin and eosin for histologic examination. Paraffin sections were also stained with an alcian blue at pH 2.5-periodic acid Schiff (PAS) sequence which distinguished blue- or purple-stained acidic mucosubstance from red-colored neutral mucosaccharide.

Trachea specimens for scanning electron microscopy were fixed with Karnovsky's paraformaldehyde-glutaraldehyde phosphate-buffered fixative. The tissues were washed in distilled water, and dehydrated with increasing concentration of alcohol. Pentyl acetate was then substituted for the alcohol, and the tissues were critical point dried in carbon dioxide. The dried specimens were cemented to a stub, gold coated by vacuum evaporation and examined in a Mark II Stereoscan scanning electron microscope at 20 kV.

## DATA ANALYSIS

Results of the experiments were subjected to statistical analyses and the significance of the observed differences reported at the 5% probability level. As appropriate, linear regression analysis, analysis of variance, Chi square test, and Student t test were used. Reciprocal antibody titers were converted to $\log_{10}$ for statistical analysis (35).

In order to eliminate animal-to-animal variability in statistical analysis of tracheal explant data, each treatment group and control were assigned two ring explants from each hamster. Experimental error was further reduced by using the same ring to determine the differences between the baseline cilia beating frequency and the beating frequency at different times following exposure. Two-way analysis of variance was used to test the hypothesis of no treatment differences for the time periods studied. Duncan's new multiple-range test and the Chi square distribution test were used to elucidate patterns of significant treatment differences.

# SECTION 5

# RESULTS AND DISCUSSION

## SHORT-TERM EXPOSURES TO ACID MIST AND CARBON PARTICULATE MIXTURES

### Toxicity

The first phase of this investigation consisted of range finding experiments to provide the concentrations of acid mist which did not result in mortalities and which could be used in the infectivity studies. Table 1 shows mortality of mice after a single 3-hr exposure to acid mist. Mortalities were observed at 600 mg/m$^3$ while at 400 mg/m$^3$ and below only one out of 360 animals died. It is interesting to note that the population (density) of mice per cage during the acid mist exposure had an effect on mortality. Whenever two mice were housed per cage the mortalities were markedly higher than when five animals were housed per cage. Similarly, the data suggest that, irrespective of the housing density, male mice were more sensitive to the acid mist than female. The mortality data for hamsters indicate that this species is more resistant to the acid mist exposure than mice.

Initial experiments were also conducted to determine the concentration and duration of exposure to sulfuric acid mist-carbon particle mixture (A-C) that could be used in the infectivity studies without resulting in mortalities due to pollutant per se. Mice and hamsters were exposed once for 3 or 6 hr to a mixture of sulfuric acid mist ranging from approximately 100 to 700 mg/m$^3$ combined with a constant concentration of 5 mg/m$^3$ carbon aerosol. The results summarized in Table 2 indicate that CF$_1$ mice were more sensitive to the exposure than the BDF$_1$ strain of mice or hamsters. The maximum concentrations of A-C that did not result in deaths in CF$_1$ mice were approximately 300 mg/m$^3$ for the 3 hr or 200 mg/m$^3$ for the 6 hr exposures.

The mortalities due to multiple exposures to A-C in mice of different ages and strains are shown in Table 3. Groups of 6- to 7-week-old and 9- to 10-week-old BDF$_1$ and CF$_1$ mice were exposed at 24-hr intervals five times for 3 hr to 100 or 200 mg/m$^3$ A-C; only minimal mortalities were observed as the result of the multiple exposures irrespective of the concentration of acid. Upon exposure to 200 mg/m$^3$ A-C, the BDF$_1$ strain mice again appeared to be somewhat more resistant than the CF$_1$ mice. In all cases, the mortalities were low, with the older mice perhaps somewhat more susceptible to the toxic effects of the A-C mixture exposure.

TABLE 1. MORTALITY OF MICE AND HAMSTERS AFTER A SINGLE 3 HR EXPOSURE TO ACID MIST

| | | | Acid Mist ($mg/m^3$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 100 | | 250 | | 400 | | 600 | |
| Species | Sex | Mice/ Cage | D/T | % | D/T | % | D/T | % | D/T | % |
| Mice | M | 2 | 0/40 | 0 | 0/40 | 0 | 0/40 | 0 | 19/40 | 48 |
| | | 5 | 0/20 | 0 | 0/20 | 0 | 0/20 | 0 | 17/61 | 28 |
| Mice | F | 2 | 0/40 | 0 | 0/40 | 0 | 1/40 | 3 | 10/40 | 25 |
| | | 5 | 0/20 | 0 | 0/20 | 0 | 0/20 | 0 | 2/20 | 10 |
| Hamsters | M | 1 | 0/24 | 0 | 0/23 | 0 | 0/24 | 0 | 1/36 | 3 |

TABLE 2. MORTALITY AMONG MALE MICE AND HAMSTERS AFTER A SINGLE EXPOSURE TO SULFURIC ACID-CARBON PARTICLE MIXTURE

| Concn., $mg/m^3$ | | Mortality | | | | | |
|---|---|---|---|---|---|---|---|
| | | Mice CF1 | | Mice BDF1 | | Hamsters | |
| Acid | Carbon | D/T | % | D/T | % | D/T | % |
| 3 hr-Exposure | | | | | | | |
| 100 | 5 | 1/53 | 2 | - | | 0/24 | 0 |
| 200 | 5 | 1/25 | 4 | - | | 0/12 | 0 |
| 300 | 5 | 0/24 | 0 | 0/48 | 0 | 0/12 | 0 |
| 400 | 5 | 4/24 | 17 | 0/36 | 0 | 0/12 | 0 |
| 500 | 5 | 12/50 | 24 | 0/44 | 0 | 0/12 | 0 |
| 700 | 5 | 5/24 | 21 | - | | 0/12 | 0 |
| 6 hr-Exposure | | | | | | | |
| 100 | 5 | 0/24 | 0 | - | | 0/12 | 0 |
| 200 | 5 | 0/24 | 0 | - | | 0/12 | 0 |
| 300 | 5 | 7/72 | 10 | 0/48 | 0 | 0/12 | 0 |
| 400 | 5 | 5/24 | 21 | 0/36 | 0 | 1/12 | 8 |
| 500 | 5 | 22/48 | 46 | 2/44 | 5 | - | |

TABLE 3. MORTALITY AFTER MULTIPLE 3-HR EXPOSURES TO TWO CONCENTRATIONS OF ACID MIST PLUS 5 $mg/m^3$ CARBON

| Species | Age (weeks) | Number of Exposures | Acid + Carbon Concn., $mg/m^3$ | |
|---|---|---|---|---|
| | | | 100 + 5<br>D/T | 200 + 5<br>D/T |
| $CF_1$ mice (M) | 7 | 1 | 1/358 | 0/192 |
| | | 2 | 0/357 | 0/192 |
| | | 3 | 1/357 | 0/192 |
| | | 4 | 2/356 | 2/96 |
| | | 5 | 6/354 | 2/94 |
| $CF_1$ mice (M) | 9 | 1 | 0/138 | 1/152 |
| | | 2 | 0/138 | 11/151 |
| | | 3 | 0/138 | 4/95 |
| | | 4 | 0/138 | 2/91 |
| | | 5 | 2/138 | 5/89 |
| $BDF_1$ mice (M) | 6 | 1 | 0/48 | 0/96 |
| | | 2 | 0/48 | 0/96 |
| | | 3 | 0/48 | 0/96 |
| | | 4 | 1/48 | 0/48 |
| | | 5 | 0/47 | 0/48 |
| $BDF_1$ mice (M) | 9 | 1 | 0/48 | 0/48 |
| | | 2 | 0/48 | 0/48 |
| | | 3 | 0/48 | 0/48 |
| | | 4 | 0/48 | 0/48 |
| | | 5 | 0/48 | 2/48 |

The 7-week-old $CF_1$ mice exposed five times to 200 mg/m$^3$ A-C showed an approximate 20% loss in body weight, while 9-week-old $CF_1$ mice lost 25% weight. The 7-week-old $CF_1$ mice exposed five times to 100 mg/m$^3$ A-C showed a 16% weight loss, whereas control mice held in the exposure chamber for 3 hr/day for 5 days at ambient condition lost only 5%. Thus, in terms of body weight loss, exposure to the pollutant mixture appears to be a considerably greater stress than the mere manipulation of the animals or deprivation of food and water during exposures.

To complete the studies of the effects of short-term multiple exposures to mixtures of acid and carbon, experiments were conducted in which 3- to 4-week-old $CD_1$ male mice were exposed for 3 hr/day, 5 days/week to aerosols containing 5 mg/m$^3$ of carbon particles only. This concentration was the same as that used in combination with all of the various acid concentrations in single or short-term multiple exposure studies. Mice were exposed 5, 10, or 20 times (1, 2 or 4 weeks) to the carbon aerosol or to ambient air with no mortality resulting in any of the groups.

## Scanning Electron Microscopy

The mortality studies were accompanied by histologic and scanning electron microscopic examination of the respiratory tract to further define the effects of acid mist and acid mist-carbon mixtures on the animals.

### Tissues from Control Mice --

Nasal cavity -- As seen in the composite scanning electron micrograph (Figure 3), the nasal cavity is composed of squamous, ciliated, and nonciliated respiratory epithelium. Starting with the external nares, the surface is covered with squamous cells, which are characterized by their rough invaginated surface and well-defined cell walls (Figure 4a). The anterior portion of the septum shows a general transition from the squamous cells of the external nares to a rough irregular surface of nonciliated cells (Figure 4b). The microvilli on these cells are generally very short and compact; however, each cell varies in number of and length of microvilli (Figure 4c). Toward mid-septum a few ciliated cells can be seen (Figure 4d), while microvilli on the nonciliated cells are becoming longer and the cell surface is becoming smoother. At the posterior end, the cell surface is smooth with a heavy population of ciliated cells (Figure 4e). Cilia are longest here, although a few nonciliated cells can be seen.

Trachea -- Both ciliated and nonciliated cell types are found in the trachea. Cilia tend to be uniform in length and microvilli tend to be short and compact (Figure 5).

Lung, bronchus and alveoli -- The cells in the bronchus are similar to those found in the trachea (Figure 6a). Alveoli have a honeycomb appearance, each alveolus being divided by a thin septum (Figure 6b). Pores are also visible as small openings in each alveolus.

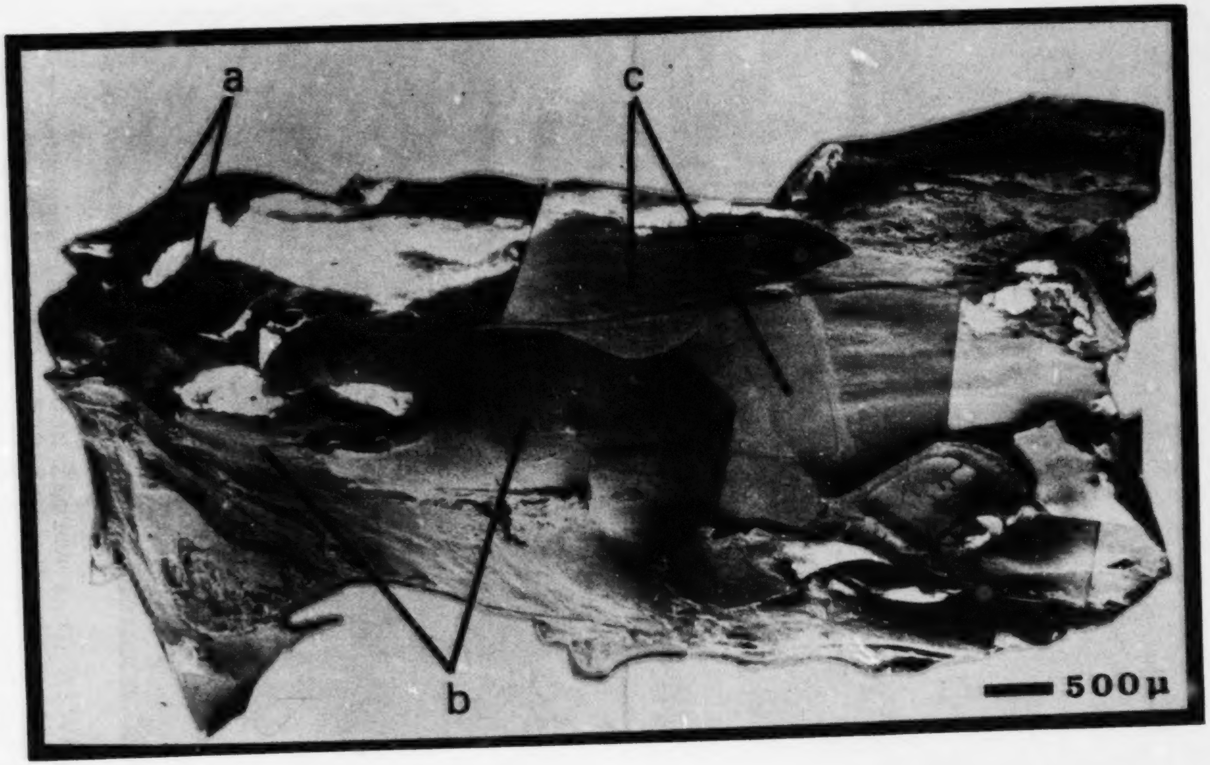

Figure 3. Composite scanning electron micrograph of the nasal cavity of a normal mouse, 20X: (a) squamous cells in external nares; (b) nonciliated cells in the anterior and mid septum; (c) ciliated cells in the mid and posterior septum.

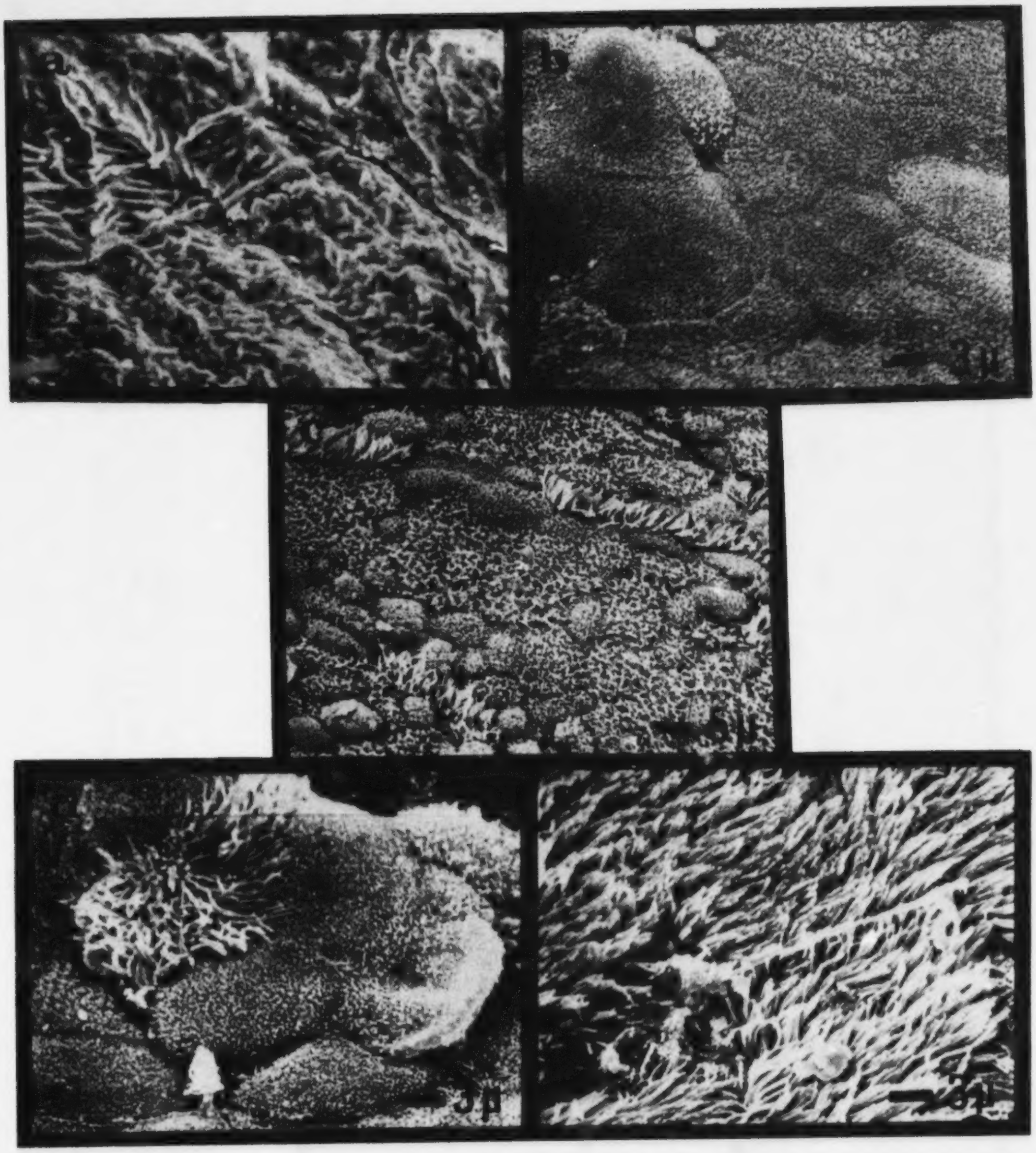

Figure 4. Nasal cavity of a normal mouse: (a) squamous cells in the external nares, 5100X; (b) nonciliated cells, 2200X; (c) nonciliated cells showing varying lengths of microvilli, 1050X; (d) nonciliated and ciliated cells of mid septum, 2100X; (e) ciliated cells at posterior septum, 2100X.

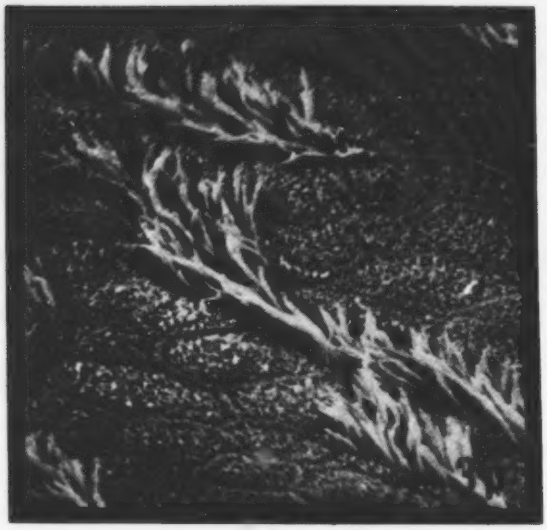

Figure 5. Trachea of a normal mouse: nonciliated and ciliated cells, 2000X.

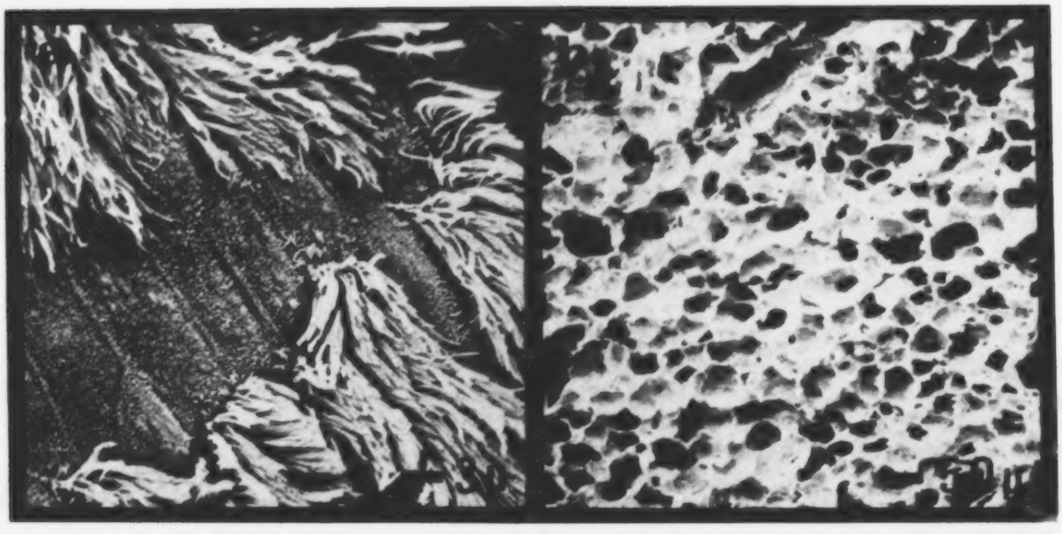

Figure 6. Lung of a normal mouse: (a) nonciliated and ciliated cells in the bronchus, 2000X; (b) alveoli, 110X.

Tissues from Mice Exposed to Acid Only and A-C --

The toxicity studies were extended to include scanning electron microscopic examination of the nasal cavity, trachea, and lung to further define the effects of exposure to A-C mixtures. Lungs from mice and hamsters exposed to 100 mg/m$^3$ acid mist for 3 hr and examined at 1, 3, 24, 48 and 72 hr past exposure did not reveal any change. The same was found true of those animals exposed to mixtures of 100 mg/m$^3$ acid mist and 5 mg/m$^3$ carbon. In animals exposed to the A-C mixtures damage was distributed throughout the entire trachea as well as down into the main stem bronchus, while in those exposed to acid mist only, the main destruction was found in the upper portion of the trachea. SEM examination of the respiratory tract following 5, 10 or 20 3-hr exposures to 5 mg/m$^3$ carbon only revealed only slightly increased sloughing of squamous cells in the external nares of animals exposed to carbon compared to the controls. The rest of the respiratory epithelium was normal.

The type of damage observed in mice exposed to various concentrations of A-C was similar in all samples, with variations in the degree of severity and the tissues involved. In general, starting at the external nares of the nasal cavity, small holes could be seen in the cell surface in the squamous cells (Figure 7a). On the anterior septum and midseptum, nonciliated cells had holes, tears, and/or missing microvilli (Figure 7b). Some dead cells began sloughing (Figure 7c) and areas could be found where a dead cell had totally sloughed off and left a hole in the surface (Figure 7d). Some ciliated cells were seen tearing away at their cell edges (Figure 7e). On the posterior septum, ciliated cells showed little damage except for a heavy mucous coating.

In mice exposed five times to 200 mg/m$^3$ A-C, or three, five and ten times to 100 mg/m$^3$ A-C, macrophages were found in the nasal cavity, especially on the anterior septum and midseptum. The highest number of macrophages was present in the group exposed to 200 mg/m$^3$ A-C where the damage was most severe and extensive, and a lesser number was found in the latter groups where the damage was less severe. These are believed to be alveolar macrophages brought up in the mucus into the nasal cavity. A mucous coating was found throughout damaged areas, being most prevalent in high damage areas and probably a result of the acid-carbon treatment.

In the trachea, a mucous coating and matted cilia were present in mice exposed to the various A-C concentrations (Figure 8a). In the trachea of some mice exposed five times to 200 mg/m$^3$ A-C, the mucous coating on the cells was so heavy that it was not possible to estimate the damage to the cell surface, while in tracheas of other mice holes, tears and dying cells were seen (Figure 8b and c), and some regeneration of cilia could be found 2 weeks after exposure (Figure 8d). In the bronchus of the lung, results were similar, i.e., mucus, matted cilia, holes, tears, and dying cells were noted.

The alveoli showed damage only when mice were exposed five times to 200 mg/m$^3$ A-C. Many of the septa dividing each alveolus were thickened and yet many emphysemic-like areas were also present where the septa were thin and filamentous (Figure 9).

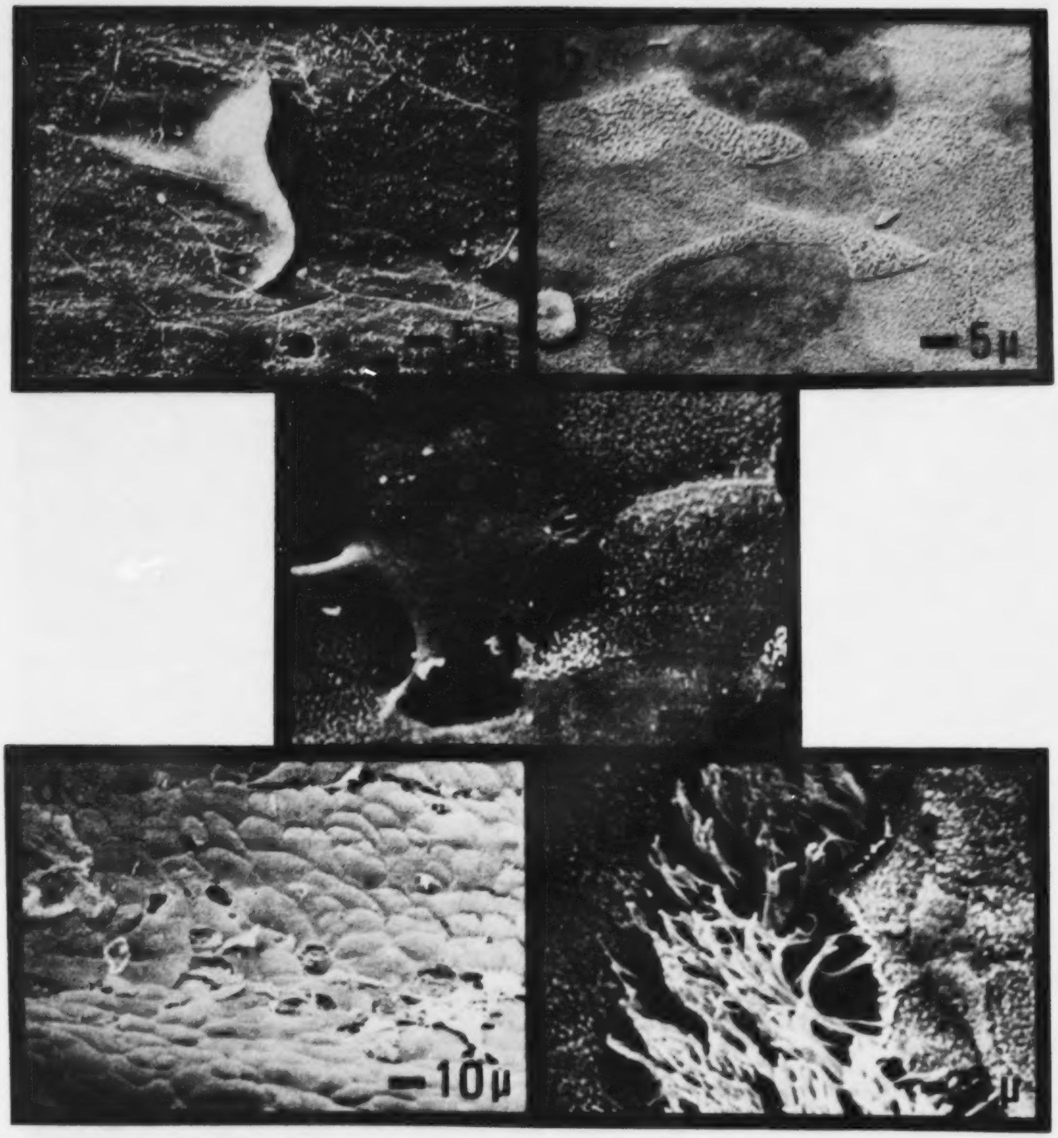

Figure 7. Nasal cavity of mouse exposed to acid mist-carbon particles mixture: (a) small holes in squamous cells, 1000X; (b) missing microvilli in nonciliated cells, 1050X; (c) damaged nonciliated cells beginning to slough off, cell shows small holes and missing microvilli, 2300X; (d) holes in cell surface where dead cells have sloughed off, 500X; (e) ciliated cell tearing away at its border on mid septum, 2000X.

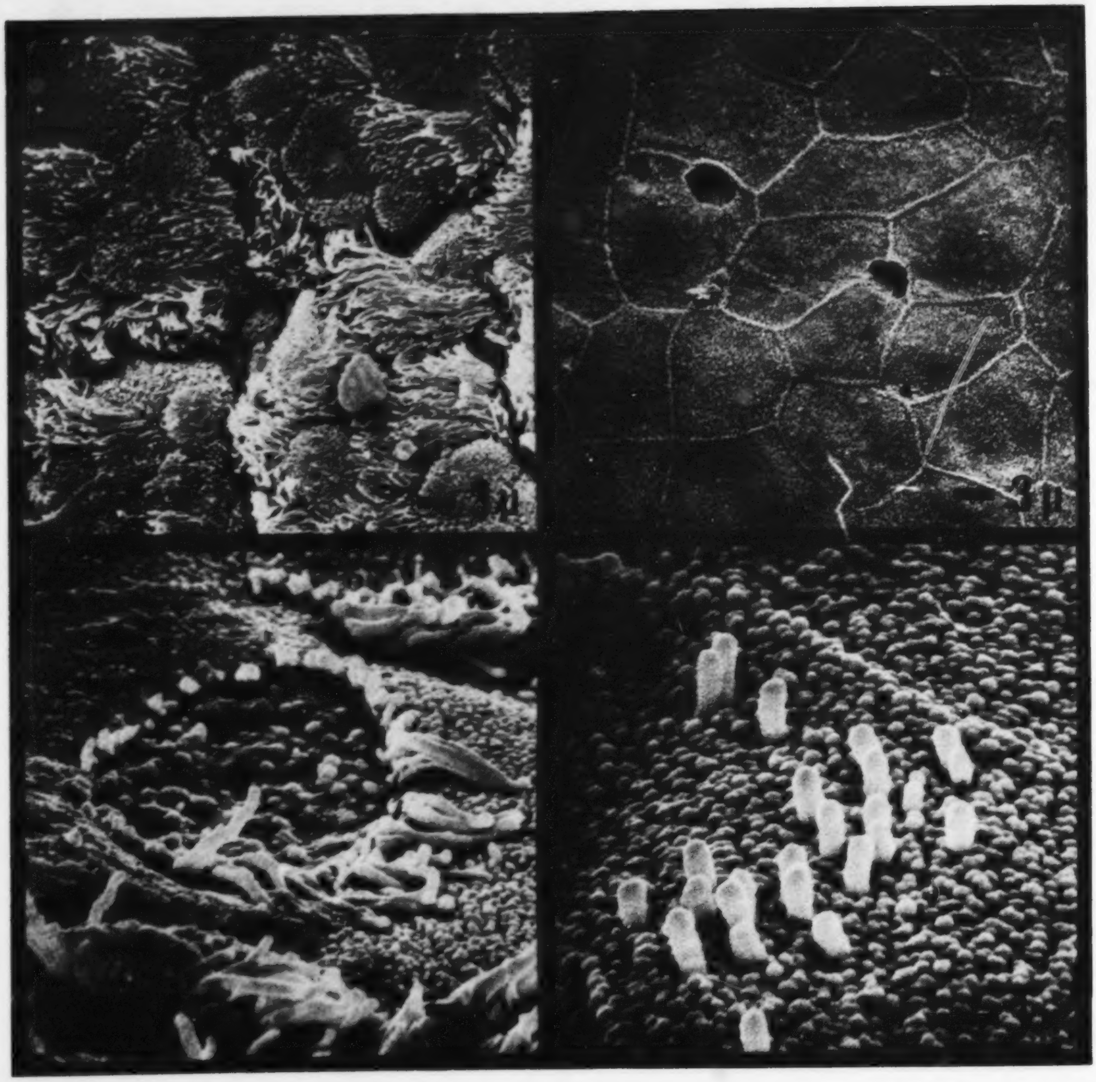

Figure 8. Trachea of mouse exposed to acid mist-carbon particles mixture: (a) mucous coating and matted cilia, 1800X; (b) holes in the cell surface of nonciliated cells, 2000X; (c) dying ciliated cell, 5200X; (d) regenerating ciliated cell at 2 weeks after exposure to the pollutants, 10,050X.

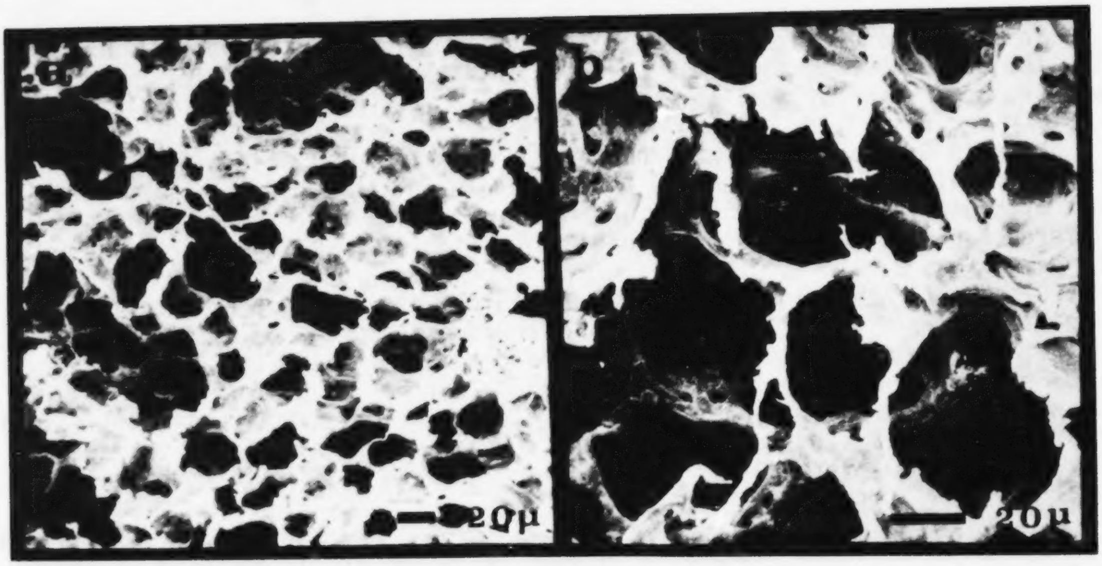

Figure 9. Lung of mouse exposed to acid mist-carbon particles mixture: thickened alveolar walls as well as thin filamentous septa (a) 200X; (b) 550X.

CT indices were calculated (Table 4) for all A-C studies so that a comparison of comparable concentrations could be evaluated. In the first four groups the amount of damage to the tissues increased as the CT for acid and the CT for carbon particles increased.

After a single exposure to 100 mg/m$^3$ of acid mist (Group I), damage was found near the top and middle of the trachea. The lower portion of the trachea was relatively free of damage and the lung was normal. When the same concentration of acid was combined with 5 mg/m$^3$ of carbon (Group II), damage was more extensive and extended into the bronchus of the lungs.

In mice exposed three or five times to 100 mg/m$^3$ A-C (Groups III and IV), the damage became more severe deeper into the bronchus. After 2 weeks, however, some signs of healing were noted.

Mice in Groups V, VII and IX were exposed to a total acid CT dose of 1000 mg/m$^3$ with carbon. From the results shown in the table it is apparent that although the total acid exposure dose was equivalent, the tissue damage differed markedly. In mice exposed ten times to 100 mg/m$^3$ A-C (Group VII) the damage was similar to that seen in mice exposed 3 or 5 times to 100 mg/m$^3$ A-C (Groups III and IV) and was much less severe than in mice exposed five times to 200 mg/m$^3$ A-C (Group V). Mice in Group V showed the most severe damage, which extended into the alveoli, and remained essentially the same at 2 weeks after the exposure. Mice exposed 20 times to 50 mg/m$^3$ A-C (Group IX), the most exposures with the lowest concentration nevertheless still equaling the CT of 1000 for acid, showed a total damage similar to those exposed to acid only (Group I). Thus, the damage here was slight despite the total exposure concentration.

By comparing the CT values for groups IV, V and VI, an increase in damage could only be seen where the acid concentration was the highest, the least damage where no acid was present, and minimal damage at 500 mg/m$^3$. Since the CT for carbon was the same in all three groups, it appeared that the concentration of acid was more important. By comparing groups VII and VIII as well as IX and X, it could also be seen that even though the CT values were equal for carbon and acid within the groups, the most damage was found in the A-C groups, the damage being more extensive in group VII where the acid concentration was the higher of the two. These observations suggest that although the carbon caused some damage, its presence served to enhance the toxicity of the acid mist. Moreover, the results demonstrated that the frequency of exposure to A-C was not as important a factor in inducing tissue damage as the initial concentration of sulfuric acid mist.

## Histopathologic Examination of Lung and Trachea Tissues

In general, tissues from experimental mice were similar to those of the controls. At the highest A-C concentration (5 daily exposures to 200 mg/m$^3$ and 5 mg/m$^3$ carbon) a slight trend toward squamous metaplasia in a few small bronchioles was apparent. Mild bronchiolar epithelial hyperplasia and bronchiolites were also present. However, very similar lesions were found in control mice and the general conclusion was that the control and experimental tissues could not be differentiated.

TABLE 4. SCANNING ELECTRON MICROSCOPIC OBSERVATIONS FROM MICE EXPOSED TO SULFURIC ACID MIST, CARBON ONLY, OR SULFURIC ACID MIST-CARBON PARTICLE MIXTURE

| Group | Concentration (mg/m$^3$) and Number of exposures (3 hr/day, 5 day/week) | CT Index Acid (A) | CT Index Carbon (C) | Tissue Nasal Cavity | Tissue Trachea | Tissue Bronchus | Tissue Lung |
|---|---|---|---|---|---|---|---|
| I | 100A x1 | 100 | - | ND | Damage heaviest near top of trachea, mucus, surface holes | Normal | Normal |
| II | (100A+5C) x1 | 100 | 5 | Mucus, holes, tears, dying cells | Damage throughout trachea, matted cilia, mucus, holes, tears | Slight damage, few dying cells | Normal |
| III | (100A+5C) x3 | 300 | 15 | Same as above, no macrophages | Same as above | Slight damage, some tears | Normal |
| IV | (100A+5C) x5 | 500 | 25 | Same as above | Same as above | Same as above | Normal |
| V | (200A+5C) x5 | 1000 | 25 | Heavy damage, mucus, holes, tears, dying cells, many macrophages | Heavy mucus, making damage assessment impossible | Dying cells, holes, tears, villi | Thickened alveoli, emphysemic-like areas |
| VI | 5C x5 | - | 25 | Slight mucus, sloughing squamous cells, rest normal | Slight mucus | Normal | Normal |

Continued

TABLE 4 (continued)

| Group | Concentration ($mg/m^3$) and Number of exposures (3 hr/day, 5 day/week) | CT Index | | Tissue | | | |
|---|---|---|---|---|---|---|---|
| | | Acid (A) | Carbon (C) | Nasal Cavity | Trachea | Bronchus | Lung |
| VII | (100A+5C) x10 | 1000 | 50 | Damage, mucus, holes, tears, dying cells, some macrophages | Damage throughout, matting of cilia, numerous holes, tears | Slight damage, some tears | Normal |
| VIII | 5C x10 | - | 50 | Slight mucus, sloughing squamous cells, rest normal | Slight mucus | Normal | Normal |
| IX | (50A+5C) x20 | 1000 | 100 | Slight damage, mucus, holes, dying cells, no macrophages | Damage throughout, matting of cilia, mucus, holes, tears | Normal | Normal |
| X | 5C x20 | - | 100 | Slight mucus, sloughing squamous cells, rest normal | Slight mucus | Normal | Normal |

## Infectious Challenge

In the infectivity studies, the combined effects of exposure to acid mists or acid-carbon mists with viral or bacterial challenge were examined. The pathogens used were influenza virus, *Klebsiella pneumoniae* and *Streptococcus* sp. Lancefield's Group C.

### Single Exposure to Acid only --

Table 5 shows the results of experiments where mice were exposed for 3-hr to 100 mg/m$^3$ acid mist and then challenged by the respiratory route with airborne *Streptococcus* or influenza virus. The interval between the acid mist exposure and the infectious challenge varied from 1 to 24-hrs. As can be seen, mortality and mean survival time did not appear to be affected by the exposure and challenge with *Streptococcus*. The extent of lung consolidation observed after the challenge with influenza virus was higher in mice exposed to acid mist than those challenged with the influenza virus only. This was especially noticable in animals challenged with the infectious agent within 1 hr after the termination of acid mist exposure, where two-fold increase in mortality and a significant increase in lung consolidation were seen.

In a similar group of experiments not shown in the table, mice were challenged with *K. pneumoniae* first and 1 to 24-hr later were exposed to 100 mg/m$^3$ of acid mist. This experimental condition did not result in any enhancement of mortality.

TABLE 5. EFFECTS OF 3-HR EXPOSURE TO 100 mg/m$^3$ ACID MIST ON RESISTANCE TO INFECTION OF MICE

| Agent | Interval Between Acid and Infect. (hr) | Mortality D/T | Mortality % | RMSR (days) | Lung Consol. |
|---|---|---|---|---|---|
| *Streptococcus* | 0[a] | 12/53 | 23 | 12.2 | - |
| | 1 | 9/45 | 20 | 12.1 | - |
| | 3 | 9/45 | 20 | 12.4 | - |
| | 24 | 11/45 | 24 | 11.8 | - |
| Influenza | 0[a] | 9/60 | 15 | 13.2 | 2.05 |
| | 1 | 16/58 | 28 | 12.6 | 2.74* |
| | 3 | 10/60 | 17 | 13.2 | 2.34 |
| | 24 | 13/55 | 24 | 12.3 | 2.57 |

* Significant difference ($p \leq 0.05$) compared to influenza virus infected control.

[a] Animals infected and not exposed to the pollutant.

In another study, hamsters were first intranasally infected with influenza virus and 72 hr later exposed to acid mist concentrations of 500 and 700 $mg/m^3$ for 3 hr. Control hamsters were given saline intranasally and then exposed to the same concentration of acid mist. As can be seen in Table 6 the exposure to acid mist per se had no effect on mortality or survival rate of the hamsters. The mortality due to infection only was 12% and increased to 27% upon exposure to 500 $mg/m^3$ and 46% upon exposure to 700 $mg/m^3$. Similarly, the survival rates were reduced from 13.1 to 84. days. A Chi square analysis performed on the data suggests a high significance of the differences ($X^2 = 6.87$). Based on this exploratory experiment, additional studies were conducted using the 72-hr interval to determine whether the mortality of mice challenged with airborne *Streptococcus* is altered by high concentration acid mist-carbon mixture exposures.

TABLE 6. MORTALITY AND SURVIVAL RATES OF HAMSTERS CHALLENGED INTRANASALLY WITH INFLUENZA VIRUS AND 72 HR LATER EXPOSED FOR 3 HR TO ACID MIST

| Agent | Acid $mg/m^3$ | Mortality D/T | Mortality % | RMSR (days) |
|---|---|---|---|---|
| Virus | 0 | 3/25 | 12 | 13.1 |
| | 500 | 3/11 | 27 | 11.0 |
| | 700 | 11/24 | 46* | 8.4* |
| Saline | 0 | 0/4 | 0 | 14 |
| | 500 | 0/5 | 0 | 14 |
| | 700 | 0/4 | 0 | 14 |

* Significant difference ($p < 0.05$) compared to influenza virus infected control.

### Single Exposure to A-C Mixtures --

Table 7 shows the mortality of mice challenged with airborne *Streptococcus* and 72 hr later exposed to A-C mixtures for 3 hr. The data show very little if any effect on mortality after the combined exposures to the pollutant and infectious challenge when compared to mortality observed in animals challenged with the *Streptococcus* only. As is seen, the exposure to peptone alone did not produce any mortalities but a few animals died after exposure to peptone followed by 3-hr exposure to the pollutant.

TABLE 7. MORTALITY OF MICE CHALLENGED WITH AIRBORNE *STREPTOCOCCUS* SP. AND EXPOSED TO ACID MIST-CARBON MIXTURES

| Agent | Acid mg/m³ | Carbon mg/m³ | Mortality D/T | Mortality % |
|---|---|---|---|---|
| *Streptococcus* | 0 | 0 | 19/102 | 19 |
| | 100 | 0 | 13/50 | 26 |
| | 100 | 5 | 9/48 | 19 |
| | 500 | 0 | 14/50 | 28 |
| | 500 | 5 | 8/48 | 17 |
| Peptone | 0 | 0 | 0/30 | 0 |
| | 100 | 0 | 1/15 | 7 |
| | 100 | 5 | 1/15 | 7 |
| | 500 | 0 | 3/15 | 20 |
| | 500 | 5 | 0/14 | 0 |

In another experiment, hamsters were used in a single acute exposure to A-C to investigate the importance of the interval between exposure to the pollutants and infectious challenge. At <1, 24, 48 or 72 hr before or after intranasal challenge with influenza A/PR/8 virus or PBS control, hamsters were exposed to 300 mg/m$^3$ and 5 mg/m$^3$ carbon for 6 hr. Results shown in Table 8 indicate that when exposure to A-C preceded the infectious challenge by <1, 24 and 72 hr, although not statistically significant, some increases in mortality and decreased survival times were noted. However, when the sequence was reversed, i.e., challenge followed by A-C exposure, significantly increased mortality was observed at the 24-hr interval and survival times decreased significantly at the 24- or 48-hr intervals.

Short-Term Multiple Exposures to A-C Mixtures --

To determine the lowest concentration and the number of repeated exposures that produce increased mortality in conjunction with either a bacterial or viral challenge, studies were initiated with infectious challenge before or after five or ten repeated 3-hr exposures to either 100 or 200 mg/m$^3$ acid mist and 5 mg/m$^3$ carbon particle mixture. Male CF$_1$ or CD$_1$ mice were used for all of these studies.

*Streptococcus* sp. -- Mice were exposed daily for 3 hr, 5 days/week for 1 and 2 weeks, to 100 mg/m$^3$ acid mist and 5 mg/m$^3$ carbon particle mixture either immediately before or after challenge with airborne *Streptococcus* sp. When the A-C exposures preceded the infectious challenges, no differences in mortality and

TABLE 8. MORTALITY AND SURVIVAL TIME OF HAMSTERS CHALLENGED INTRANASALLY WITH INFLEUNZA A/PR/8 VIRUS BEFORE OR AFTER A SINGLE 6-HR EXPOSURE TO 300 mg/m$^3$ ACID MIST AND 5 mg/m$^3$ CARBON MIXTURE

| Experimental Sequence | Challenge Agent | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A/PR/8 | | | | | PBS | | |
| | Mortality | | | RMSR (days) | P | Mortality | | RMSR (days) |
| Interval Between A-C Exposure and Challenge (hr) | D/T | % | P | | | D/T | % | |
| Influenza-air control | 8/40 | 20 | | 12.2 | | 0/28 | 0 | 14.0 |
| <1 | 11/41 | 27 | N.S. | 11.3 | N.S. | 1/27 | 4 | 13.8 |
| 24 | 11/36 | 31 | N.S. | 11.2 | N.S. | 0/25 | 0 | 14.0 |
| 48 | 7/42 | 17 | N.S. | 12.5 | N.S. | 0/28 | 0 | 14.0 |
| 72 | 13/42 | 31 | N.S. | 11.2 | N.S. | 0/27 | 0 | 14.0 |
| Interval Between Challenge and A-C Exposure (hr) | | | | | | | | |
| Influenza-air control | 11/44 | 25 | | 11.9 | | 0/26 | 0 | 14.0 |
| <1 | 9/44 | 20 | N.S. | 12.4 | N.S. | 0/26 | 0 | 14.0 |
| 24 | 23/44 | 52 | 0.009 | 8.1 | 0.0003 | 0/26 | 0 | 14.0 |
| 48 | 19/44 | 43 | 0.07 | 9.3 | 0.007 | 2/26 | 8 | 14.0 |
| 72 | 16/44 | 36 | N.S. | 10.6 | 0.09 | 3/26 | 12 | 13.9 |

RMSR were noted (Table 9). Reversing the sequence (infection followed by A-C exposure) both five and ten exposures produced an increase in mortality which was more marked when the number of A-C exposures increased. When the concentration of pollutant was increased to 200 mg/m$^3$ acid mist and 5 mg/m$^3$ carbon particle mixture, a significant increase in mortality and decreased survival time compared to infected controls occurred with both pollutant exposure/ infection sequences. These data correlate with the SEM observations indicating more severe lung damage in mice exposed five times to 200 mg/m$^3$ than ten times to 100 mg/m$^3$ A-C.

Out of a total of 188 mice serving as controls challenged with 0.1% peptone water aerosol and exposed to either A-C or air, 11 died (6%). In the reverse experimental sequence no death occurred among mice challenged with peptone water after exposure to A-C or air.

Influenza virus -- Studies were initiated in which five daily 3-hr exposures to 100 mg/m$^3$ acid mist and 5 mg/m$^3$ carbon particle mixture were combined with challenge with influenza A$_2$/Taiwan virus aerosol at <1, 24, 48 or 72 hr before or after the A-C exposure. The 24- and 48-hr periods were included because these intervals resulted in most marked mortality increases when a single 6-hr exposure to 300 mg/m$^3$ acid mist and 5 mg/m$^3$ carbon particle mixture was combined with influenza virus challenge in hamsters. The results shown in Table 10 indicate a significant increase in mortality and decrease in mean survival time of mice exposed to the pollutants in the 24-hr interval experiments. Slightly increased mortalities were also seen when the infectious challenge occurred 1 or 72 hr after the final A-C exposure, but these increases were not statistically significant. Among control mice used in the various sequence studies, 3 out of 287 died (1%).

A similar series of experiments was conducted using airborne influenza A$_2$/Taiwan virus as the challenge agent immediately before or after five daily 3-hr exposures to 200 mg/m$^3$ acid mist and 5 mg/m$^3$ carbon particle mixture. In both sequences of challenge and pollutant exposures, a significant decrease in survival time and significantly increased mortality in A-C exposed infected groups was observed when compared with ambient controls (Table 10).

Another condition which produced a CT value of 1000 was twenty daily exposures to 50 mg/m$^3$ acid mist. Mice were exposed 3 hr/day, 5 days/week, for 4 weeks to 50 mg/m$^3$ acid mist and 5 mg/m$^3$ carbon particle mixture and challenged by the respiratory route with influenza A$_2$/Taiwan virus aerosol within 1 or 24 hr after the final pollutant exposure. The results (Table 11) show that, in both time-interval groups, the mortality increased and mean survival time decreased compared with ambient controls. The control mortality in the 24-hr interval study was quite high, but some increase was nevertheless detected in the experimental mice. The increase in mortality in the <1-hr interval study was statistically significant. In addition, survival time was significantly reduced in both experimental groups. Lung consolidation in the <1-hr interval experiment increased significantly in the experimental mice as compared to ambient controls.

TABLE 9. RESPONSE OF MICE EXPOSED 3 HR TO ACID MIST AND CARBON PARTICLE MIXTURE AND CHALLENGED WITH *STREPTOCOCCUS* SP.

| | Multiple exposures to acid concentration + 5 mg/m$^3$ carbon | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 100 mg/m$^3$ | | | | | | | | | | 200 mg/m$^3$ | | | | |
| | 5X | | | | | 10X | | | | | 5X | | | | |
| | Mortality | | | RMSR | | Mortality | | | RMSR | | Mortality | | | RMSR | |
| Experimental Sequence[a] | D/T | % | P | days | P | D/T | % | P | days | P | D/T | % | P | days | P |
| Air/Strep | 18/59 | 31 | | 11.5 | | 14/86 | 16 | | 12.6 | | 10/80 | 13 | | 12.9 | |
| A-C/Strep | 19/60 | 32 | N.S. | 11.6 | N.S. | 18/80 | 23 | N.S. | 12.1 | N.S. | 22/79 | 28 | 0.02 | 10.7 | 0.001 |
| Strep/air | 16/58 | 28 | | 12.0 | | 10/48 | 21 | | 12.1 | | 61/274 | 22 | | 12.1 | |
| Strep/A-C | 21/58 | 36 | N.S. | 10.8 | 0.07 | 18/48 | 38 | 0.07 | 10.6 | 0.04 | 109/274 | 40 | 0.00001 | 10.5 | 0.00005 |

[a] Interval between infectious challenge and exposure to the pollutants was $\leq 1$ hr.

TABLE 10. RESPONSE OF MICE TO 5 DAILY 3-HR EXPOSURES TO ACID MIST AND CARBON PARTICLE MIXTURE AND CHALLENGED WITH INFLUENZA $A_2$/TAIWAN VIRUS

| Experimental Exposure | | | | Mortality | | | | | RMSR (days) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Concn., mg/m$^3$ | | Exposure | Interval[a] | Control | | Exp. | | | | | |
| Acid | Carbon | Sequence | (hr) | D/T | % | D/T | % | P | Control | Exp. | P |
| 100 | 5 | A-C/virus | <1 | 14/80 | 18 | 19/80 | 24 | N.S. | 13.1 | 12.9 | N.S. |
| | | | 24 | 5/168 | 3 | 16/165 | 10 | 0.01 | 13.9 | 13.5 | 0.07 |
| | | | 48 | 16/167 | 10 | 16/164 | 10 | N.S. | 13.6 | 13.5 | N.S. |
| | | | 72 | 16/80 | 20 | 20/79 | 25 | N.S. | 12.8 | 12.9 | N.S. |
| 200 | 5 | A-C/virus | <1 | 10/96 | 10 | 20/90 | 22 | 0.03 | 13.6 | 11.8 | 0.0003 |
| 100 | 5 | Virus/A-C | <1 | 4/40 | 10 | 4/40 | 10 | N.S. | 13.4 | 13.1 | N.S. |
| | | | 24 | 14/168 | 2 | 29/166 | 17 | 0.01 | 13.6 | 12.8 | 0.0008 |
| | | | 48 | 23/168 | 14 | 25/168 | 15 | N.S. | 13.4 | 13.4 | N.S. |
| | | | 72 | 9/40 | 23 | 4/40 | 10 | N.S. | 13.0 | 13.6 | N.S. |
| 200 | 5 | Virus/A-C | <1 | 13/108 | 12 | 55/108 | 51 | 0.00001 | 13.1 | 10.8 | 0.00001 |

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[a] Interval between A-C exposure and infectious challenge.

TABLE 11. RESPONSE OF MICE TO 20 DAILY EXPOSURES (3 HR/DAY, 5 DAYS/WEEK FOR 4 WEEKS) TO 50 mg/m3 ACID MIST AND 5 mg/m$^3$ CARBON PARTICLE MIXTURE AND CHALLENGED WITH INFLUENZA $A_2$/TAIWAN VIRUS

| Interval Between A-C Exposure and Challenge (hr) | Mortality |  |  |  |  | RMSR |  |  | Pulmonary Consolidation |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | Cont. |  | Exp. |  |  | (days) |  |  |  |  |  |
|  | D/T | % | D/T | % | P | Cont. | Exp. | P | Cont. | Exp. | P |
| <1 | 12/59 | 20 | 23/58 | 40 | 0.02 | 13.1 | 11.8 | 0.005 | 2.6 | 3.4 | 0.004 |
| 24 | 40/58 | 69 | 45/59 | 76 | N.S. | 10.1 | 9.1 | 0.03 | Not Done |  |  |

Short-Term Multiple Exposures to Carbon Only --

Limited experiments were also conducted in which 3- to 4-week-old CD1 male mice were exposed to aerosols containing 5 mg/m$^3$ of carbon particles only. This concentration was the same as that used in combination with all of the various acid concentrations in single or short-term multiple exposures.

Mice were challenged with airborne influenza A$_2$/Taiwan virus within 1 hr before or after 5, 10 or 20 daily exposures to 5 mg/m3 carbon, 3 hr/day, 5 days/week. The results in Table 12 show that there were no changes in mortality, survival time or pulmonary consolidation in mice exposed 5 or 10 times to the carbon aerosol either before or after virus challenge. However, the mice challenged with virus after 20 exposures to the carbon aerosol had significantly increased mortality and pulmonary consolidation and decreased mean survival time compared to the controls.

TABLE 12. RESPONSE OF MICE EXPOSED TO 5 mg/m$^3$ CARBON AEROSOL AND CHALLENGED WITH INFLUENZA A$_2$/TAIWAN VIRUS

| Challenge Sequence | Number of 3-hr Carbon Exposures | Mortality D/T | Mortality % | RMSR (days) | Pulmonary Consolidation |
|---|---|---|---|---|---|
| Pre-carbon exposure | 5 | 0/48 | 0 | 14.0 | 1.44 |
| | 10 | 1/48 | 2 | 13.9 | 1.56 |
| | Air-Control | 2/48 | 4 | 13.9 | 1.40 |
| Post-carbon exposure | 10 | 6/48 | 12.5 | 12.4 | 2.58 |
| | 20 | 11/48 | 23* | 12.3* | 3.02* |
| | Air-Control | 3/48 | 6.25 | 12.8 | 2.38 |

* Significant difference ($p < 0.05$) compared to air control.

## Bacterial Clearance Rate

The clearance rates of viable *Streptococcus* sp. from lungs of mice exposed to several different concentrations of A-C were examined. The t½ values representing the time in hours required to clear 50% of viable bacteria from the lungs were based on results of two replicate experiments. As seen in Table 13, control mice held in air cleared bacteria more rapidly than any of the A-C groups. Moreover, the increase of t½ values appeared to be related to increase in A-C concentration or the number of exposures.

TABLE 13. CLEARANCE OF VIABLE *STREPTOCOCCUS* FROM LUNGS OF MICE EXPOSED FOR 3 HR TO ACID MIST AND CARBON PARTICLE MIXTURE

| Exposure | | | t½ (hr) | |
|---|---|---|---|---|
| Concn., mg/m$^3$ | | Number of Exposures | | |
| Acid | Carbon | | | Control |
| 100 | 5 | 1 | 1.35 | 1.31 |
| 300 | 5 | 1 | 1.56 | 1.26 |
| 200 | 5 | 5 | 1.58 | 1.04 |

A single 3-hr exposure to 100 mg/m$^3$ acid mist and 5 mg/m$^3$ carbon particle mixture did not alter the rate of clearance of viable bacteria from the lungs. This paralleled with absence of changes in mortality due to bacterial respiratory infection in response to a single 3-hr exposure to 100 mg/m$^3$ A-C. Upon a single 3-hr exposure to 300 mg/m$^3$ acid mist and 5 mg/m$^3$ carbon particle mixture, the highest A-C concentration that did not result in toxicity deaths, the t½ was somewhat increased. Five daily 3-hr exposures to 200 mg/m$^3$ A-C produced a much greater increase in the time required to clear 50% of viable bacteria from the lungs. For this exposure condition significant increases in mortality due to bacterial respiratory infection have been found, demonstrating a relationship between the efficacy of clearance of viable bacteria from the lung and the ability of mice to survive an infectious challenge. It should be noted that markedly fewer bacteria were initially recovered (0 hr) from lungs of mice exposed 5 times to 200 A-C than from control mice. Hence, it is apparent that mice exposed to A-C inhaled fewer bacteria, but were not able to clear them as efficiently as control mice which initially inhaled more viable bacteria.

## Alveolar Macrophages

The specific capacity of the AM to handle inhaled bacteria is the result of many factors including their metabolic activity, immunologic experience, and the presence of pulmonary or other disease. In addition, certain environmental factors have been shown experimentally to impair the functional state of the cells, thus pulmonary antibacterial activity. Among these are hypoxia, ethanol, acute starvation, corticosteroids, progressive renal disease, and gaseous air pollutants (36).

Since phagocytosis by AM is considered to be an important defense mechanism, the number and the activity of these cells in lung washings have been used as an indication of the capability of lungs to deal with foreign particles.

Mechanisms of clearance of viable bacteria from the lung include physical removal of the bacterial material by the mucociliary apparatus and lymphatic drainage system, and the phagocytosis which destroys the viability of the organism, with or without removal from the lung. A number of studies (32, 37-42) have shown that physical removal of bacteria deposited in the lung accounts for only a small fraction of the total reduction in viable counts recovered over a given period. Therefore the loss of viable microorganisms is primarily a result of *in situ* bactericidal activity.

Green and Goldstein (32) described a method to measure *in vivo* inactivation of staphylococci in the lungs of mice by simultaneous determination of physical removal and bactericidal activity. They quantitated intrapulmonary bacterial inactivation in individual animals, rather than by the conventional method determining group mean bacterial clearance. Animals are exposed to radiolabeled live bacteria and the ratio of the viable bacterial count to the radioactive count in each animal's lung provides the rate at which bacteria are destroyed by the lung in a given time after infection. Decline in the radioactive count in the lungs gives the rate of physical removal of bacteria by the mucociliary system.

Rylander (42) used this technique to investigate the effects of exposure to coal dust, sulfur dioxide ($SO_2$), and a combination of both on the pulmonary bactericidal activity and on physical removal of inhaled bacteria in guinea pigs. Exposure to 26.2 mg/m$^3$ (10 ppm) $SO_2$ for four weeks did not affect the bacterial elimination mechanisms, whereas 15 mg/m$^3$ carbon black reduced the bactericidal capacity with the implication that the capacity of the mechanisms of phagocytosis had decreased. When the two agents were given simultaneously they produced a synergistic effect decreasing the mucous flow. In studies involving other air pollutants, it was found that exposure to ozone and $NO_2$ alone, and in combination, resulted in significant decreases in pulmonary bactericidal activity (43-45) and no synergistic effect.

For this program, a limited number of experiments was conducted to examine the effect of sulfuric acid-carbon mixtures on the cellular defense system in the lungs of mice. In all of these experiments single exposures of 3 hr duration were used at concentrations of 100 mg/m$^3$ of sulfuric acid and 5 mg/m$^3$ carbon. Two approaches were taken: in the first, after exposure to A-C mist the mice were killed and alveolar macrophages lavaged from their lungs were examined for total and differential cell counts, viability, *in vitro* phagocytic activity, and cell surface morphology. In the second approach, after A-C exposure the mice were infected with *S. aureus* by intranasal inoculation and bacterial inactivation was determined in the lungs.

Results on alveolar macrophage examinations are summarized in Table 14. The table lists the viability, total cell count and phagocytic index values determined on alveolar macrophages lavaged less than 1 hr and 24 hr after the 3 hr exposure to A-C mixtures. There are no changes in viability but a slight trend toward decreased cell counts and increased phagocytic indices in macrophages of mice could be observed immediately as well as 24 hr after the exposure. Differential counts, not shown in the table, were in the range of 97-98% alveolar macrophages with polymorphonuclear leukocytes and lymphocytes under 2% and were not affected by the exposure.

Examination of the effect of A-C exposure on the surface structure of alveolar macrophages showed that compared with air controls there were pronounced changes in macrophages lavaged in less than 1 hr after A-C exposure, as shown in Figures 10a through f.

TABLE 14. VIABILITY, TOTAL CELL COUNT AND PHAGOCYTIC INDEX OF ALVEOLAR MACROPHAGES LAVAGED FROM MICE EXPOSED FOR 3 HR TO ACID-CARBON MIST[a]

| Interval Between Acid-Carbon Mist and Lavage | Viability,% | | Total Cell Count x $10^5$ | | Phagocytic Index, % | |
|---|---|---|---|---|---|---|
| | Air | A-C | Air | A-C | Air | A-C |
| <1 hr | 93.9 | 93.3 | 9.1 | 8.2 | 12.4 | 14.0 |
| 24 hr | 93.9 | 93.7 | 9.6 | 8.5 | 10.1 | 13.5 |

[a] 100 mg/m$^3$ $H_2SO_4$ and 5 mg/m$^3$ carbon.

Figure 10a and b show macrophages typical of those obtained from control mice. The intricate surface structure with numerous surface processes of the cells extending in all directions can be seen especially clearly at higher magnification. In the control samples between 90 and 100% of the cells had similar surface structure. In Figure 10c macrophages lavaged from mice less than 1 hr after 3 hr exposure to 100 mg/m$^3$ acid and 5 mg/m$^3$ carbon are seen. There are distinct changes in surface structure. The cells are excessively spread out on the glass substrate, the surface processes have atrophied and distinct holes can be seen in one of them. The damage is shown in more detail in Figure 10d at higher magnification, while in Figure 10e a macrophage in the transitional stage is shown: some of the normal surface structure is still retained in the center, but it is increasingly spread and flattened out near the periphery.

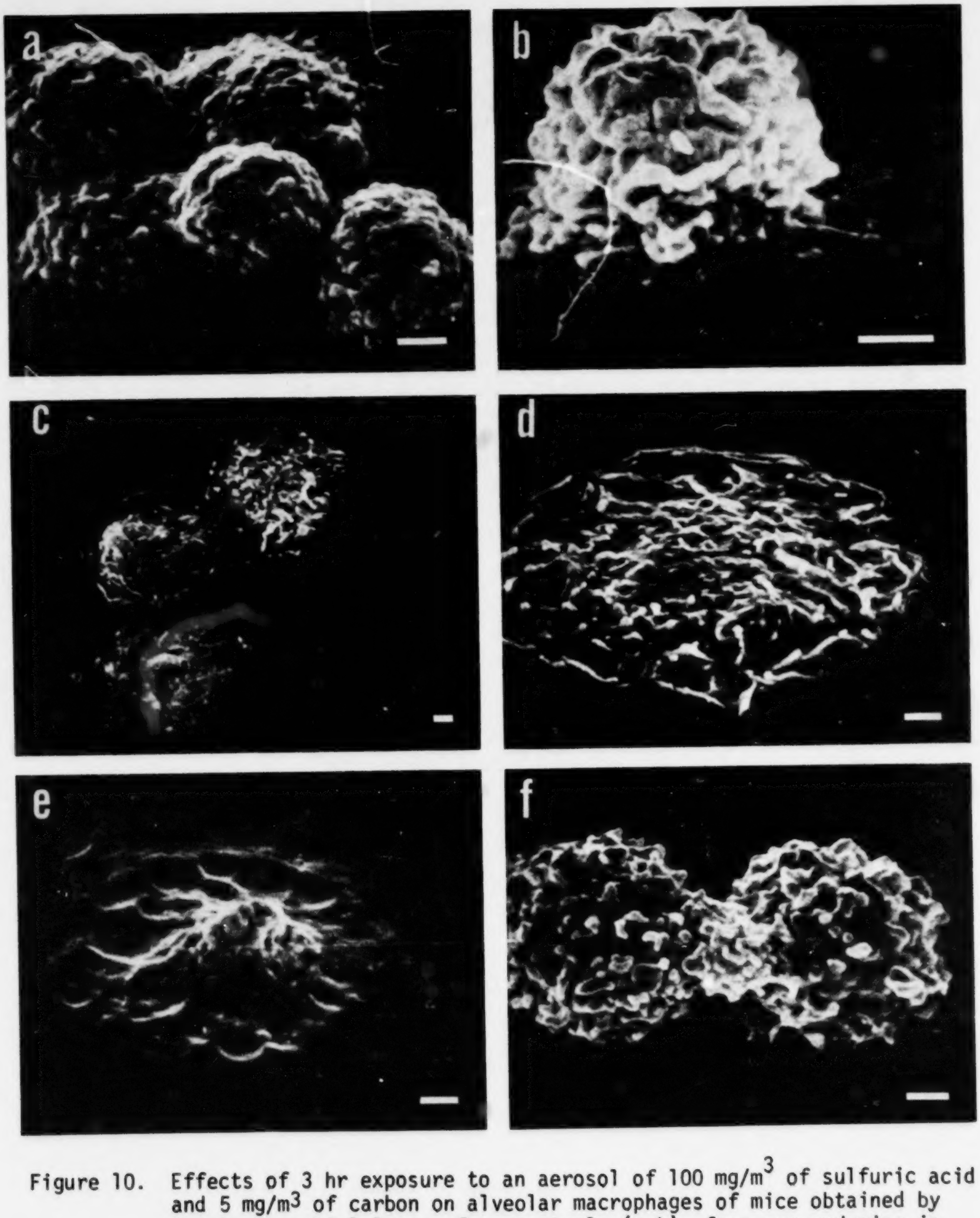

Figure 10. Effects of 3 hr exposure to an aerosol of 100 mg/m$^3$ of sulfuric acid and 5 mg/m$^3$ of carbon on alveolar macrophages of mice obtained by tracheobronchial lavage from controls (a,b), from exposed mice in less than 1 hr after exposure (c,d,e) and 24 hr after exposure (f). The bar in the lower right hand corner represents 2 μ.

Counts made to obtain an estimate of the altered cell population in the A-C exposed samples compared to the controls showed that 78% of the macrophages had these distinctly different surface structural features.

When macrophages were lavaged from mice 24 hr after A-C exposure these changes were no longer present and the morphology (shown in Figure 10f) as well as the percent distribution (96%) was similar to the unexposed controls.

Bactericidal activity in the lungs of mice challenged with *S. aureus* less than 1 hr and 24 hr after A-C exposure is shown in Table 15. The number of mice used ranged from 25 to 30 per exposure group. The intranasally administered dose of *S. aureus* was 1 x $10^6$ bacteria in 0.05 ml of 0.1% peptone suspension; the number of bacteria actually deposited in the lungs, as determined by radioactive counts, ranged from 3.5 to 7.0 x $10^5$. The results show a significant increase in the percent of bacteria remaining, reflecting decrease in bactericidal activity, in lungs of mice infected with *S. aureus* immediately after exposure to A-C. However, mice infected 24 hr after the exposure cleared the bacteria at a rate equal to that of the corresponding controls. These data support the scanning electron microscopic observation of morphologic alterations in the macrophages which were seen immediately after A-C exposure but not 24 hr later.

TABLE 15. BACTERICIDAL ACTIVITY IN LUNGS OF MICE EXPOSED FOR 3 HR TO SULFURIC ACID - CARBON[a] MIST

| Interval Between A-C Mist and *S. aureus* Challenge | Bacteria Remaining (%) in Lungs of Mice Exposed to: Air |  | A-C |  |
|---|---|---|---|---|
|  | Geom. Mean | SE | Geom. Mean | SE |
| <1 hr | 25.7 | 3.8 | 33.1* | 4.0 |
| 24 hr | 30.9 | 3.8 | 30.9 | 3.8 |

[a] 100 mg/$m^3$ $H_2SO_4$ and 5 mg/$m^3$ carbon.

* Significant difference (<0.05) compared to control.

## Vaccine-Induced Immunity

The effects of exposure on preformed antibody as well as formation of antibody during A-C exposure were examined using vaccine-induced immunity. Briefly, 5- to 6-week-old male $CD_1$ mice were vaccinated subcutaneously with approximately 370 CCA units of influenza $A_2$/Japan/170 virus vaccine. Control mice received subcutaneous injections of PBS. Before or after vaccination the mice were exposed daily for 3 hr, 5 days/week to 100 mg/$m^3$ acid mist and 5 mg/$m^3$ carbon particle mixture or ambient air. At 2, 8 and 16 weeks after vaccination, one group of mice, consisting of 20 animals/group, was killed to determine HI and SN antibody levels. The remaining mice were challenged by the respiratory route with infectious influenza $A_2$/Japan/170 virus. Two weeks after challenge, the surviving animals were killed, lung consolidation scored, and sera collected for serological assay to allow comparison of primary and secondary response. All sera were pooled by groups of two.

At 2 weeks after vaccination (Table 16), exposure to A-C had no effect on the secondary SN antibody response obtained following infectious challenge. This is consistent with the mortality, survival time, and pulmonary consolidation data which similarly showed no difference between vaccinated mice exposed to air or A-C. However, the secondary HI antibody response was significantly depressed. At 8 weeks after vaccination, exposure to A-C caused a marked depression of the secondary SN antibody response following infectious challenge, although the HI antibody response was unchanged. Similarly, although the mortality and survival time were not affected, there was a significant increase in pulmonary consolidation in mice exposed to A-C compared to the ambient controls. At 16 weeks after vaccination, A-C exposure resulted in slightly higher secondary HI and SN antibody titers, but no change in the other parameters.

The effects of A-C mists on antibody formation are shown in Tables 17 and 18. Exposure to A-C or ambient air occurred immediately before or after vaccination. At 2, 8, and 16 weeks after vaccination, sera were collected and all remaining mice challenged with infectious homologous virus. In both studies, there was generally no effect on mortality nor change in the primary SN or HI antibody response when measured 2 weeks after vaccination or in the secondary SN antibody titers following infectious challenge. However, a significant decrease in lesions and significantly depressed secondary HI titers were seen when vaccination was followed by A-C exposure (Table 17), while significantly increased pulmonary consolidation was observed when vaccination was preceded by A-C exposure (Table 18). The primary SN antibody titers measured 8 weeks after vaccination were, in both cases, significantly higher in mice exposed to A-C. However, this pattern was not paralleled by the primary HI antibody response which did not change significantly. Following infectious challenge, the secondary SN antibody response was significantly depressed in mice which had been exposed to A-C immediately before vaccination (Table 18), although no changes were observed in the other parameters. In

TABLE 16. RESPONSE OF MICE VACCINATED WITH INFLUENZA $A_2$/JAPAN VIRUS, CHALLENGED WITH HOMOLOGOUS VIRUS AND IMMEDIATELY EXPOSED TO FIVE DAILY 3-HR DOSES OF 100 mg/m$^3$ ACID MIST AND 5 mg/m$^3$ CARBON

| Interval[a] (wk) | Experimental Sequence | Mortality | | RMSR (days) | Pulmonary Consolidation | | Geometric Mean Reciprocal Antibody Titer | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Primary Response[b] | Secondary Response[b] | | Primary Response[b] | Secondary Response[b] | |
| | | D/T | % | | Score | P | SN | SN | P | HI | HI | P |
| 2 | Nonvaccinated air controls | 41/117 | 35 | 11.8 | 2.9 | | <8 | 32 | | <8 | 9 | |
| | Vac/virus/air | 0/76 | 0 | 14.0 | 0.8 | | <8 | 324 | | <8 | 79 | |
| | Vac/virus/A-C | 1/76 | 1 | 14.0 | 0.7 | N.S. | c | 325 | N.S. | c | 39 | 0.01 |
| 8 | Nonvaccinated air controls | 32/80 | 40 | 11.4 | 3.1 | | <8 | 35 | | <8 | 18 | |
| | Vac/virus/air | 0/80 | 0 | 14.0 | 0.6 | | 28 | 372 | | <8 | 69 | |
| | Vac/virus/A-C | 0/80 | 0 | 14.0 | 1.1 | 0.0002 | c | 324 | 0.06 | c | 69 | N.S. |
| 16 | Nonvaccinated air controls | 53/87 | 61 | 10.0 | 3.7 | | <8 | 24 | | <8 | 20 | |
| | Vac/virus/air | 1/79 | 1 | 14.0 | 0.8 | | <8 | 375 | | <8 | 85 | |
| | Vac/virus/A-C | 1/75 | 1 | 14.0 | 0.9 | N.S. | c | 457 | N.S. | c | 105 | N.S. |

[a] Interval between vaccination and infectious challenge.

[b] In nonvaccinated mice, "primary immune response" is that antibody titer prior to virus challenge and "secondary immune response" is antibody titer after virus challenge.

[c] Primary immune response is the same for mice exposed to either air or A-C since animals were bled just prior to challenge to virus and exposure to A-C.

TABLE 17. RESPONSE OF MICE VACCINATED WITH INFLUENZA $A_2$/JAPAN VIRUS, IMMEDIATELY EXPOSED TO FIVE DAILY 3-HR DOSES OF 100 $mg/m^3$ ACID MIST AND 5 $mg/m^3$ CARBON, THEN CHALLENGED WITH HOMOLOGOUS VIRUS

| Interval[a] (wk) | Experimental Sequence | Mortality | | | RMSR | | Pulmonary Consolidation | | Geometric Mean Reciprocal Antibody Titer | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Primary Response[b] | | Secondary Response[b] | | Primary Response[b] | Secondary Response[b] | |
| | | D/T | % | P | (days) | P | Score | P | SN | P | SN | P | HI | HI | P |
| 2 | Nonvaccinated air controls | 100/139 | 72 | | 8.8 | | 4.0 | | <8 | | 44 | | <8 | 20 | |
| | Vac/air/virus | 1/57 | 2 | | 13.8 | | 1.7 | | <8 | | 427 | | <8 | 112 | |
| | Vac/A-C/virus | 0/68 | 0 | N.S. | 14.0 | N.S. | 1.0 | 0.00002 | <8 | N.S. | 324 | N.S. | <8 | 59 | 0.05 |
| 8 | Nonvaccinated air controls | 29/79 | 37 | | 11.9 | | 2.8 | | <8 | | 21 | | <8 | 30 | |
| | Vac/air/virus | 0/79 | 0 | | 14.0 | | 0.5 | | 15 | | 229 | | 14 | 40 | |
| | Vac/A-C/virus | 0/76 | 0 | N.S. | 14.0 | N.S. | 0.8 | 0.005 | 23 | 0.06 | 257 | N.S. | 19 | 59 | N.S. |
| 16 | Nonvaccinated air controls | 66/87 | 76 | | 8.8 | | 4.3 | | <8 | | 17 | | <8 | 37 | |
| | Vac/air/virus | 0/68 | 0 | | 14.0 | | 1.0 | | 9 | | 776 | | 12 | 209 | |
| | Vac/A-C/virus | 7/77 | 9 | 0.04 | 13.3 | 0.01 | 1.5 | 0.01 | 8 | N.S. | 631 | N.S. | 10 | 170 | N.S. |

[a] Interval between vaccination and infectious challenge.

[b] In nonvaccinated mice, "primary immune response" is that antibody titer in mice prior to virus challenge and "secondary immune response" is antibody titer after virus challenge.

TABLE 18. RESPONSE OF MICE EXPOSED TO FIVE DAILY 3-HR DOSES OF 100 mg/m$^3$ ACID MIST AND 5 mg/m$^3$ CARBON, IMMEDIATELY VACCINATED WITH INFLUENZA A2/JAPAN VIRUS, AND CHALLENGED WITH HOMOLOGOUS VIRUS

| Interval[a] (wk) | Experimental Sequence | Mortality |  | RMSR (days) | Pulmonary Consolidation |  | Geometric Mean Reciprocal Antibody Titer |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  |  |  | Primary Response[b] |  | Secondary Response[b] |  | Primary Response[b] | Secondary Response[b] |  |
|  |  | D/T | % |  | Score | P | SN | P | SN | P | HI | HI | P |
| 2 | Nonvaccinated air controls | 46/79 | 58 | 10.3 | 3.8 |  | <8 |  | 30 |  | <8 | 8 |  |
|  | Air/vac/virus | 0/80 | 0 | 14.0 | 0.5 |  | 8 |  | 229 |  | <8 | 83 |  |
|  | A-C/vac/virus | 0/80 | 0 | 14.0 | 0.8 | 0.03 | <8 | N.S. | 234 | N.S. | <8 | 102 | N.S. |
| 8 | Nonvaccinated air controls | 67/89 | 75 | 9.3 | 4.2 |  | <8 |  | 36 |  | <8 | 23 |  |
|  | Air/vac/virus | 0/80 | 0 | 14.0 | 0.8 |  | 12 |  | 891 |  | 11 | 155 |  |
|  | A-C/vac/virus | 0/82 | 0 | 14.0 | 0.9 | N.S. | 25 | 0.03 | 575 | 0.03 | 10 | 132 | N.S. |
| 16 | Nonvaccinated air controls | 60/90 | 67 | 9.5 | 3.9 |  | <8 |  | 38 |  | <8 | 28 |  |
|  | Air/vac/virus | 0/77 | 0 | 14.0 | 1.5 |  | 16 |  | 912 |  | <8 | 257 |  |
|  | A-C/vac/virus | 0/82 | 0 | 14.0 | 1.8 | 0.0025 | 16 | N.S. | 631 | 0.11 | <8 | 110 | 0.002 |

[a] Interval between vaccination and infectious challenge.

[b] In nonvaccinated mice, "primary immune response" is that antibody titer in mice prior to virus challenge and "secondary immune response" is antibody titer after virus challenge.

addition, the HI antibody response was somewhat depressed. Both HI and SN secondary responses were similar when the A-C exposure followed immediately the vaccination. Mortality and survival times were unchanged, but lung consolidation scores were significantly higher in the A-C exposure group. At 16 weeks after vaccination, there were no differences in the primary SN or HI antibody responses between the A-C or ambient exposure groups. Secondary antibody titers following infectious challenge were slightly depressed in all A-C exposure groups, the HI response significantly so in mice exposed to A-C immediately before vaccination. Lung consolidation was also significantly increased in both experimental groups.

In summary, 2 weeks after vaccination, the primary immune response ranged from <8 to 8. Nevertheless, the vaccinated mice, whether exposed to air or A-C mists, were protected to the same extent against infectious challenge and showed good HI and SN secondary responses. Only the lung consolidation and secondary HI antibody data indicated differences between experimental and control mice in this time period. No pattern was noted with lung consolidation. However, the secondary HI response was signifcantly depressed when A-C exposure followed immediately after vaccination. This was the same study in which lung consolidation was significantly depressed. When A-C exposure preceded vaccination, the significant increase in pulmonary consolidation was accompanied by a slight increase in secondary HI antibody response.

At 8 weeks after vaccination, a primary immune response was noted with considerably higher SN titers in mice exposed to A-C mists. The secondary HI and SN response in the experimental mice were in general similar to the vaccinated air controls. Only when A-C preceded vaccination was the SN secondary response significantly depressed compared to the vaccinated air control (Table 18).

By 16 weeks after vaccination, no effects were noted on preformed antibody (Table 16). However some slight depression in the secondary response was still present, as was a significantly increased pulmonary consolidation and a significant increase in mortality in mice vaccinated, immediately exposed to A-C mists, and 16 weeks later challenged with infectious virus. Significantly increased pulmonary consolidation and marked decreases in both HI and SN secondary antibody responses were noted in mice exposed to A-C mists immediately before vaccination (Table 18).

In general, both the HI and SN antibody levels showed changes in the same direction.

## Hamster Tracheal Organ Culture

Hamster tracheal organ culture was used to assess the effects of sulfuric acid mist, carbon particles and mixtures of A-C on ciliary activity and alterations in tracheal epithelium. Two approaches were used, namely *in vivo* and *in vitro* exposure to the pollutants. Different combinations of *in vivo* and *in vitro* exposure and/or maintenance were also examined to determine the correlation between *in vivo* exposure damage and recovery and the effects observed in organ culture.

### *In vivo* Exposure and Maintenance --

Tracheas from hamsters exposed for 3 hr to a range of 0.8-1.4 mg/$m^3$ sulfuric acid mist alone, mist combined with 1.1-1.9 mg/$m^3$ carbon, and 1.1-1.9 mg/$m^3$ carbon only were removed and cut transversely into a series of rings immediately after and 24, 48, and 72 hr after exposure. Alterations in cilia beating frequency were observed,and the rings were processed for histological and scanning electron microscopic examinations. Each experiment was performed in duplicate.

The data shown in Table 19 indicate that the means for the carbon treatment group did not differ significantly from controls throughout the study. The mean ciliary beating frequency for sulfuric acid mist alone and acid mist-carbon measured immediately and 24 hr after exposure was significantly lower than controls. At 48 and 72 hr the means of the acid mist treatment group were significantly lower than the controls, carbon alone,and acid mist-carbon.

TABLE 19. CILIARY BEATING FREQUENCY IN TRACHEAS FROM HAMSTERS AT <1, 24, 48 AND 72 HR AFTER TERMINATION OF A 3-HR *IN VIVO* EXPOSURE TO SULFURIC ACID MIST AND CARBON PARTICLES

| Concn., mg/$m^3$ | | Ciliary Beat Frequency<br>Time after Exposure, hour | | | |
|---|---|---|---|---|---|
| $H_2SO_4$ | Carbon | <1 | 24 | 48 | 72 |
| 0 | 0 | $1190^{a*}$ | $1186^{a}$ | $1177^{a}$ | $1190^{a}$ |
| 0 | 1.5 | $1190^{a}$ | $1155^{ab}$ | $1166^{a}$ | $1180^{a}$ |
| 1.1 | 0 | $1101^{b}$ | $1107^{b}$ | $1027^{b}$ | $1090^{b}$ |
| 1.1 | 1.5 | $1122^{b}$ | $1106^{b}$ | $1133^{a}$ | $1147^{a}$ |

* Identical superscripts *do not* differ significantly from each other (Duncan's New Multiple-Range Test).

Immediately after exposure to sulfuric acid mist-carbon the tracheal epithelium showed significantly greater cytological alteration than when exposed to air, carbon, and air mist alone (Table 20). The pattern of damage to the ciliated epithelium seen in the acid mist group was similar but less severe than that seen after exposure to acid mist-carbon. The general appearance of the epithelium no longer had its sharp outline; it was uneven and had a swollen appearance. A number of epithelial cells appeared in clusters protruding into the lumen. Immediately after exposure to carbon some ciliary disorganization and, in some cases, loss of cilia and rounding and sloughing of epithelial cells were seen. Tracheal epithelium of hamsters exposed to carbon and acid-mist alone showed continuous recovery throughout the 72-hr period. By 72 hr after exposure, only tracheal epithelium from the A-C treatment group still showed significant damage as compared to the ambient air control.

TABLE 20. PERCENTAGE OF NORMAL EPITHELIUM OF TRACHEAS FROM HAMSTERS AT <1, 24, 48 AND 72 HR AFTER TERMINATION OF A 3-HR EXPOSURE TO SULFURIC ACID MIST AND CARBON PARTICLES

| Concn., $mg/m^3$ | | Percentage of Normal Epithelium* Time in Culture, hour | | | |
|---|---|---|---|---|---|
| $H_2SO_4$ | Carbon | <1 | 24 | 48 | 72 |
| 0 | 0 | $91^{a*}$ | $94^{a}$ | $97^{a}$ | $97^{a}$ |
| 0 | 1.5 | $74^{b}$ | $84^{ab}$ | $94^{a}$ | $97^{a}$ |
| 1.1 | 0 | $63^{b}$ | $70^{b}$ | $75^{b}$ | $84^{ab}$ |
| 1.1 | 1.5 | $43^{c}$ | $46^{c}$ | $50^{c}$ | $72^{b}$ |

[*] Normal epithelium is defined as a smooth luminal surface with beating cilia.

[**] Identical superscripts *do not* differ significantly from each other (Chi square Distribution Test).

Histopathological examinations of normal hamster trachea immediately after removal from the animal revealed a layer of pseudostratified, ciliated, columnar epithelium, with a single layer of basal cells over a relatively thin lamina propria and submucosa. The epithelium of specimens exposed to carbon exhibited moderate pathological alterations during the 72 hr observation period. The surface of the tracheal epithelium 24 hr after exposure showed focal loss of ciliated cells which became more diffuse over the next 48 hr. Exfoliation resulting from dying cells resulted in some flattening of the epithelium. With the alcian blue-PAS sequence, a moderate PAS reactivity demonstrative of a high concentration of mucosubstance was observed. In tracheas removed <1 hr after exposure to acid mist alone and A-C, severe loss of ciliated cells in focal areas was observed. By 72 hr after exposure, the epithelium was composed of one to three layers of basal cells, goblet cells and a severe loss of ciliated cells in focal areas. A marked increase in PAS positive (acid) mucosubstance was observed at 72 hr.

SEM examinations of tracheas of animals exposed *in vivo* to carbon or acid mist alone, or acid mist-carbon showed similar epithelial surface alterations as tracheas exposed *in vivo* and maintained *in vitro*. Description of these alterations will be described in the following section (*In vivo* Exposure; *In vitro* Maintenance).

These data indicate that acid mist alone and acid mist in combination with carbon particulates produce a significant cytotoxic effect in hamster tracheal epithelium. The overall damage, i.e., depression of cilia beat frequency and damage to the normal columnar orientation of the cells in the epithelium, produced by the A-C mixture was greater than that produced by the acid mist or carbon alone. This again indicates ciliary beating frequency alone is an insufficient indicator of assessing toxicity and should be combined with other observations and measurements.

*In vivo* Exposure; *In vitro* Maintenance --

The effects of sulfuric acid mist (0.8 to 1.8 mg/m$^3$) combined with carbon (1.1 to 1.9 mg/m$^3$) as well as acid mist (0.8 to 1.8 mg/m$^3$) or carbon (1.1 to 1.9 mg/m$^3$) only were studied in hamster tracheal epithelium maintained *in vitro*. Tracheas were removed immediately after a single 3-hr exposure to the pollutants and processed as ring organ cultures.

Immediately after removal the decline in cilia beating frequency for all pollutant treatment groups (carbon and acid mist alone, and A-C mixture) was significant when compared to ambient air control cultures (Table 21). Within 24 hr after exposure, the ciliary beat frequency of the carbon and A-C treatment groups returned to control levels. At 24 hr only the mean of the acid mist group was significantly different from the control. The acid mist group showed a significant recovery at 48 hr following exposure.

TABLE 21. CILIARY BEATING FREQUENCY IN TRACHEAL RING CULTURES HELD *IN VITRO* AT <1, 24, 48 AND 72 HR AFTER TERMINATION OF A 3 HR *IN VIVO* EXPOSURE TO SULFURIC ACID MIST AND CARBON PARTICLES

| Concn., mg/m$^3$ | | Ciliary Beat Frequency<br>Time after Exposure, hour | | | |
|---|---|---|---|---|---|
| $H_2SO_4$ | Carbon | <1 | 24 | 48 | 72 |
| 0 | 0 | 1204$^{a*}$ | 1180$^{a}$ | 1155$^{a}$ | 1168$^{a}$ |
| 0 | 1.5 | 1137$^{b}$ | 1129$^{ab}$ | 1134$^{a}$ | 1144$^{a}$ |
| 1.1 | 0 | 1075$^{b}$ | 1094$^{b}$ | 1103$^{a}$ | 1129$^{a}$ |
| 1.3 | 1.5 | 1119$^{b}$ | 1116$^{ab}$ | 1119$^{a}$ | 1132$^{a}$ |

* Identical superscripts *do not* differ significantly from each other (Duncan's New Multiple-Range Test).

The amount of damage to the epithelial layer immediately after exposure to carbon and acid mist alone, or A-C combination was significantly different from the ambient air controls (Table 22). Significant cytological alterations continued in the acid mist and A-C groups throughout the 72 hr observation period. Twenty-four hr after exposure, the cytological alterations in both acid mist and A-C exposed groups were significantly different from either the control or carbon treated groups. Morphological changes observed by light microscopy resembled those described in the section on *in vivo* exposure and maintenance.

Tracheal ring organ cultures also proved applicable to the examination of the recovery from *in vivo* exposure to sulfuric acid mist and/or carbon. Tracheas maintained in organ culture following exposure to acid mist or A-C mixtures showed similar recovery patterns during the 72 hr observation period. Organ cultures exposed to carbon only had significant alterations in the ciliated epithelium compared to the controls, but were normal after 24 hr. With the exception of one or two observations, this trend of recovery was similar to that observed in the *in vivo* experiments previously described.

Pathological changes in the control and pollutant treatment groups during the 72 hr observation period were also similar to those described in the *in vivo* test system. There was a moderate loss of ciliated cells in focal areas of the epithelium of tracheas exposed to acid mist and A-C mixture. After 72 hr in culture, the cellular composition of tracheal epithelium was the same as described for *in vitro* maintenance. The histochemical properties indicated moderate increase in PAS reactivity (acidic mucosubstances).

TABLE 22. PERCENTAGE OF NORMAL EPITHELIUM OF TRACHEAL RING CULTURES HELD *IN VITRO* AT <1, 24, 48 AND 72 HR AFTER TERMINATION OF A 3 HR *IN VIVO* EXPOSURE TO SULFURIC ACID MIST AND CARBON PARTICLES

| Concn., mg/$m^3$ | | Percentage of Normal Epithelium[*] | | | |
|---|---|---|---|---|---|
| | | Time in Culture, hour | | | |
| $H_2SO_4$ | Carbon | <1 | 24 | 48 | 72 |
| 0 | 0 | $94^{a}$[**] | $86^{a}$ | $88^{a}$ | $90^{a}$ |
| 0 | 1.5 | $73^{b}$ | $75^{a}$ | $87^{a}$ | $87^{ab}$ |
| 1.3 | 0 | $56^{c}$ | $56^{b}$ | $62^{b}$ | $74^{b}$ |
| 1.3 | 1.5 | $50^{c}$ | $52^{b}$ | $62^{b}$ | $75^{b}$ |

[*] Normal epithelium is defined as a smooth luminal surface with beating cilia.

[**] Identical superscripts *do not* differ significantly from each other (Chi Square Distribution Test).

Hamster tracheal epithelial surface consists of three distinct cell types, ciliated, microvillous and goblet cells, in that order of frequency, when examined by scanning electron microscopy. Ambient air controls fixed immediately after removal from the animal showed evidence of cilia growth. In control explants fixed at 24 and 48 hr in culture, cilia buds were being formed and fully formed ciliated cells were present. In controls fixed after 72 hr in culture, only fully developed ciliated cells were observed. There were areas, however, where ciliated cells were almost completely absent. In these areas microvillous cells were the predominant cell type. Hamster tracheal epithelium removed and fixed immediately after exposure to carbon showed a network-like structure with some mucus secretions covering the surface. At 24 hr, cilia buds were present. The network-like structure was still visible along with areas of sparsely ciliated cells. In cultures removed 48 hr after exposure to carbon, good growth of cilia similar to that observed in the ambient air controls could be found. At 72 hr, nonciliated cells were seen interspersed within a layer of ciliated cells. Tracheal epithelium observed immediately after exposure to sulfuric acid mist alone or with carbon showed a pattern similar to that from carbon exposed animals except the damage was more severe. Large areas of the network-like structures could be seen in conjunction with only a few ciliated cells. In tracheal explants 24 hr after exposure, the network-like structure was still present but ciliary buds were visible. The acid mist and A-C groups showed new growth of ciliated cells along with heavily ciliated areas 48 hr following exposure. Areas of dead cells with missing microvilli were also visible. In tracheal explants 72 hr after exposure, new growth of cilia was present but it was not as extensive as that observed in ambient air control or carbon treatment groups. The observations of epithelial damage by scanning techniques are comparable to those described above in the histological and cytological studies.

The results indicate that inhalation of acid mist in combination with carbon, or acid mist or carbon alone, significantly depresses the cilia beating frequency and damages normal ciliated epithelium. The damage produced by A-C mixtures did not differ significantly from that produced by acid mist only, but it was greater than that observed after exposure to carbon alone.

*In vitro* Exposure and Maintenance --

Studies were conducted to determine the concentration of sulfuric acid that produces epithelial damage *in vitro* similar to that obtained from *in vivo* exposure. Such microscopic alterations in the tracheal epithelium were observed with a 1:$10^6$ dilution of concentrated sulfuric acid. Tracheal ring explants were exposed for 3 hr to this dilution of sulfuric acid, 150 μg/ml of carbon and an A-C combination. Dishes containing the pollutants were incubated in a 5% $CO_2$ atmosphere chamber, placed on a rocker platform rocking 10 cycles/min. Explants were exposed in 2 ml of simple medium (saline and 0.05% glucose). A pH of 6.05 ± 0.1 was maintained throughout the 3 hr exposure.

A decline in ciliary activity was observed at <1, 24, 48 and 72 hr after exposure (Table 23). The ciliary beating frequency of A-C group immediately after exposure was significantly reduced from the control (no pollutant) and acid and carbon alone. At 24 hr the means of both acid alone and A-C mixture groups were significantly lower. By 48 hr after exposure to acid and A-C, recovery from damage was evident and persisted through 72 hr.

The pattern of damage to the epithelium was similar to that in tracheal tissue observed after *in vivo* exposure. Epithelial cells were swollen and appeared in clumps, giving the luminal surface a moth-eaten appearance. Damage resulting from exposure to carbon, acid and A-C could be detected <1 hr after the 3-hr exposure period (Table 24). This persisted for 24 hr. At 48 hr after exposure to A-C or acid only, damage to the organ culture was still evident. Recovery was seen 72 hr after exposure in all treatment groups.

Histopathological and scanning electron microscopic alterations similar to those seen and described in the section titled "*In Vivo* Exposure; *In Vitro* Maintenance" were also present in the tracheal organ cultures exposed to sulfuric acid and carbon alone, or A-C mixture. As in the *in vivo* exposure, sulfuric acid alone and the A-C mixture produced similar effects. Recovery of the epithelium from the A-C mixture again was similar to that observed in explants exposed to acid alone. Carbon-exposed explants recovered to a level similar to the controls. Statistically, there was a clear correlation between initial damage to tracheal cells in organ culture and their recovery, and the ability to reproduce the effects observed in hamsters after *in vivo* exposure.

TABLE 23. CILIARY BEATING FREQUENCY IN TRACHEAL RING CULTURES AT <1, 24, 48 AND 72 HR AFTER TERMINATION OF A 3-HR *IN VITRO* EXPOSURE TO SULFURIC ACID MIST AND CARBON PARTICLES

| | | Ciliary Beat Frequency | | | |
|---|---|---|---|---|---|
| | | Time after Exposure, hour | | | |
| $H_2SO_4$ (dilution) | Carbon (μg/ml) | <1 | 24 | 48 | 72 |
| 0 | 0 | $1195^{a*}$ | $1191^{a}$ | $1143^{a}$ | $1128^{a}$ |
| 0 | 150 | $1140^{ab}$ | $1135^{ab}$ | $1144^{a}$ | $1131^{a}$ |
| $1{:}10^6$ | 0 | $1146^{ab}$ | $1112^{b}$ | $1115^{a}$ | $1106^{a}$ |
| $1{:}10^6$ | 150 | $1101^{b}$ | $1091^{b}$ | $1107^{a}$ | $1111^{a}$ |

[*] Identical superscripts *do not* differ significantly from each other (Duncan's New Multiple-Range Test).

TABLE 24. PERCENTAGE OF NORMAL EPITHELIUM IN TRACHEAL RING CULTURES AT <1, 24, 48 AND 72 HR AFTER TERMINATION OF A 3-HR *IN VITRO* EXPOSURE TO SULFURIC ACID AND CARBON PARTICLES

| | | Percent of Normal Epithelium* | | | |
|---|---|---|---|---|---|
| | | Time in Culture, hour | | | |
| $H_2SO_4$ (dilution) | Carbon (μg/ml) | <1 | 24 | 48 | 72 |
| 0 | 0 | $88^{a**}$ | $84^{a}$ | $89^{a}$ | $90^{a}$ |
| 0 | 150 | $72^{b}$ | $69^{ab}$ | $74^{ab}$ | $80^{a}$ |
| $1{:}10^6$ | 0 | $57^{b}$ | $61^{b}$ | $71^{b}$ | $78^{a}$ |
| $1{:}10^6$ | 150 | $59^{b}$ | $63^{b}$ | $70^{b}$ | $75^{a}$ |

[*] Normal epithelium is defined as a smooth luminal surface with beating cilia.

[**] Identical superscripts *do not* differ significantly from each other (Chi Square Distribution Test).

The question was raised as to whether the damage to the tracheal ciliated epithelium was due to sulfuric acid itself or whether the alterations were pH related. To answer this question, hydrochloric acid in sufficient dilution to adjust the pH of a saline and a 0.05% glucose medium to 5.0 was made. Explants were exposed to this medium for 3 hr and observed with the phase microscope for ciliostasis or gross exfoliation. Hydrochloric acid sufficient to reduce the pH to 5.0 caused approximately 50 to 75% ciliary arrest but did not alter normal cellular morphology. When explants were transferred to fresh culture medium (CMRL 1066), the cilia resumed beating at a normal frequency. These results indicated that pH 5.0 produced no lasting morphological effects and that other factors are responsible for toxicity produced by sulfuric acid.

## LONG-TERM EXPOSURE TO CARBON AND A-C MIXTURE

Long-term low concentration studies were conducted to determine the effect of exposure to carbon or A-C mixtures on normal (uninfected) mice and on the response to challenge with infectious influenza virus. Male $CD_1$ mice, 4 to 5 weeks old at the initiation of the study were exposed 3 hr/day, 5 days/week to air, carbon only (mean concentration ± standard deviation, $1.5 \pm 0.4$ $mg/m^3$ carbon) or A-C ($1.4 \pm 0.4$ $mg/m^3$ acid mixed with $1.5 \pm 0.4$ $mg/m^3$ carbon). Body weight and temperature data were collected weekly from the same mice from each exposure condition throughout the study. To determine immunoglobulin concentrations a separate group of mice was bled orbitally after 1, 4, 12, and 20 weeks of exposure. Additional parameters studied after 4, 12, and 20 weeks exposure of uninfected mice were Jerne plaque assay for number of plaque-forming cells in the spleen; examination of the respiratory tract by SEM and conventional microscopy; examination of total cell counts and viability of alveolar macrophages (AM) lavaged from the lungs; and examination of *in vivo* bactericidal capacity in the lungs. Examination of the effects of 4, 12, and 20 weeks exposure to the pollutant on the host's response to challenge with infectious influenza virus included observation of mortality, mean survival time and lung consolidation.

### Body Weight and Temperature

During the 20 weeks of pollutant exposure 24 individually marked mice in each exposure group (air, carbon, and A-C) were weighed weekly and rectal temperatures recorded. All three groups were similarly caged and deprived of food and water during the 3-hr exposures. The mean body temperatures ranged between 36.9 and 38.1°C and those of experimental mice were not significantly different from the air controls. All three groups of mice gained weight consistently throughout the 20 weeks. However, the average weight of mice exposed to A-C was consistently lower than the air controls, with mice exposed to carbon intermediate (Figure 11). All three groups were essentially the same weight at the beginning of the study. The mice exposed to the pollutant showed an initial lag in weight gain, but eventually approximated weights of control mice. The 20 week exposure interval can be divided into two phases related to the age of the mice: the interval up to 8 weeks representing a rapid growth and from 9 to 20 weeks during which growth rate of control mice decreased and average body weights reached a plateau. Regression analysis shows a markedly lower rate of growth in both the carbon and A-C groups during the initial rapid growth period.

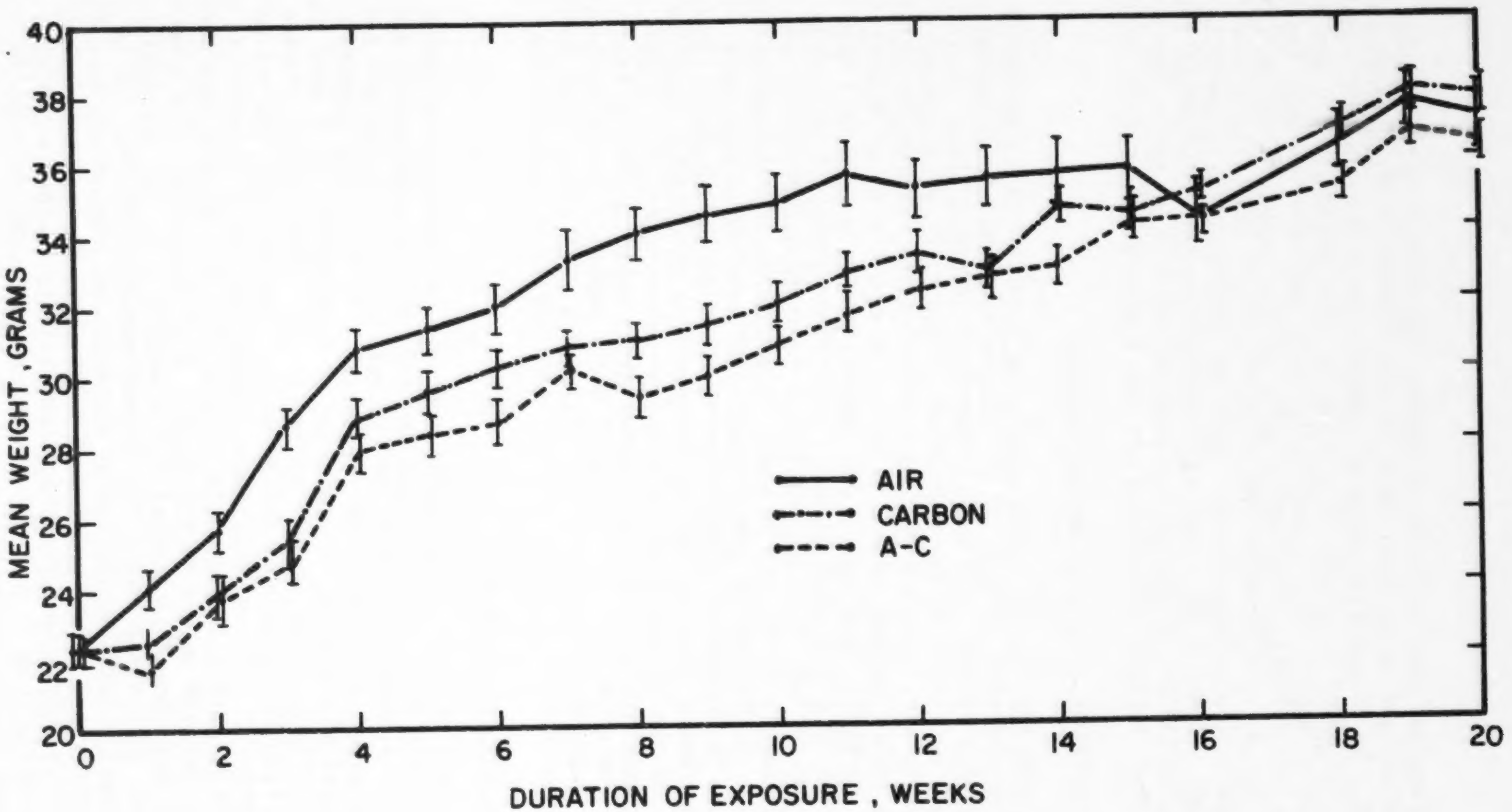

Figure 11. Body weights of mice exposed to air, carbon only (1.5 mg/m$^3$ carbon), or A-C (1.4 mg/m$^3$ sulfuric acid mist and 1.5 mg/m$^3$ carbon) 3 hr/day, 5 days/week for 20 weeks.

## Tissue Toxicity

Nasal cavities (Figure 12), tracheas (Figure 13a,b) and lungs (Figure 13c, d,e,f) from experimental and control animals were prepared for SEM and conventional histopathologic examinations. Subtle changes in the respiratory epithelium were detected by SEM examination of tissues collected after the final pollutant exposure. The normal respiratory tract surface as seen by SEM was described previously in this report and by others (46).

At 12 weeks exposure to carbon only, more squamous cells than normal were seen sloughing at the external nares (Figure 12b), but the remaining nasal epithelium was normal. Tracheas showed many mucous cells and some dying cells with loss of microvilli. Cells in the bronchus appeared normal. However, some areas of congestion were found in the alveoli. These appeared as uneven raised areas at low magnification (Figure 13d). At higher magnification the areas appeared to be thickened and fused alveoli with enlarged alveolar pores (Figure 13f). Such areas were never found in the control animals (Figures 13c,e).

Although similar, the damage was somewhat more severe in animals exposed to A-C for 12 weeks. As in the carbon group, an increased number of sloughing cells was seen at the external nares. Some non-ciliated cells were dying (Figure 12d) or had small holes around their edges. Dying cells in the trachea had small holes around the cell borders (Figure 13b). Bronchus cells were normal, but alveoli were fused as in samples from mice exposed to carbon only. Such areas were more extensive in animals exposed to A-C than in carbon-exposed animals.

At 20 weeks the damage was similar to that seen at 12 weeks, but to a lesser degree. In both A-C and carbon-exposed animals, squamous cells were sloughing more than usual, but the remaining respiratory epithelium was normal. Tracheas showed some mucus, but no damage. Lung bronchus cells were normal. Lung alveoli showed the same fusing as before, but to a lesser degree.

Thus, subtle changes were detected by SEM in the epithelium of the respiratory tract of mice after long-term exposure to carbon or A-C. In general, no changes were seen at 4 weeks. At 12 weeks, increased sloughing of squamous cells, some dying cells in the nasal cavity, and fused and thickened lung alveoli were seen in both A-C and carbon-exposed animals. In addition, holes were seen around the borders of the dying cells in the nasal cavities of mice exposed to A-C. Some dying cells with holes were also seen in tracheas from these animals. At 20 weeks, sloughing cells in the nasal cavity and fused and thickened alveoli were similar in both experimental groups. The changes were less extensive than at 12 weeks.

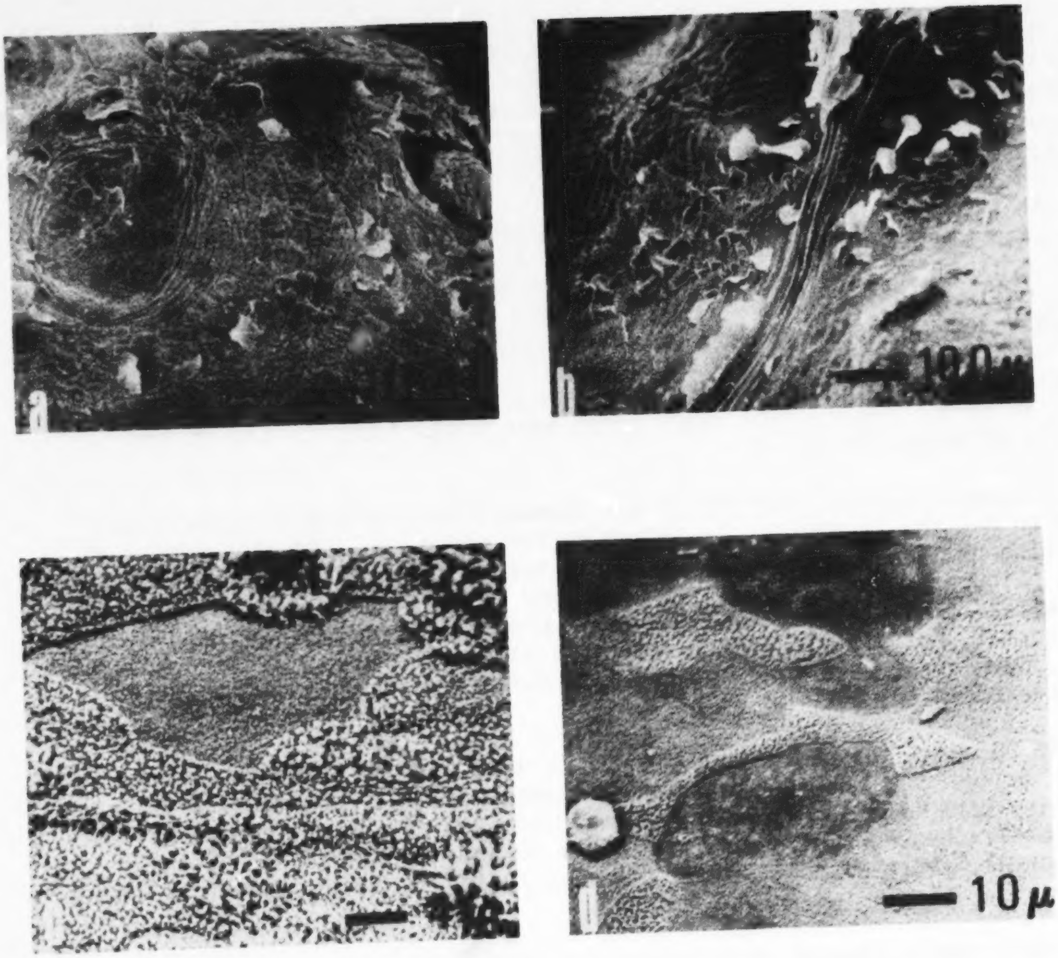

Figure 12. Nasal cavity of control and test mice. (a) sloughing of squamous cells at external nares in normal mice; (b) increased sloughing of squamous cells after 12 weeks exposure to carbon; (c) midseptum area showing ciliated cells and microvilli of normal non-ciliated cells; (d) dying non-ciliated cells showing loss of microvilli after 12 weeks exposure to A-C.

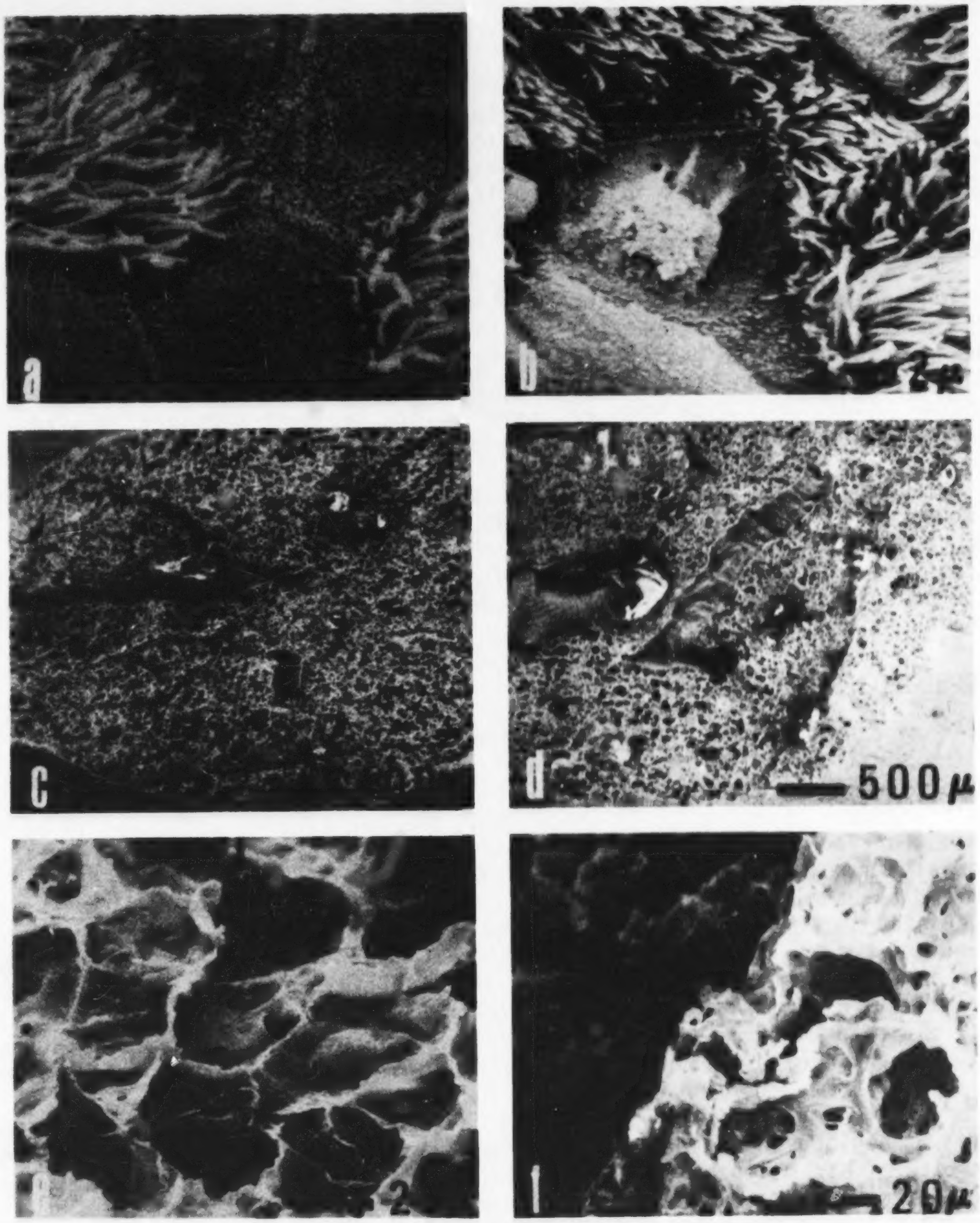

Figure 13. Trachea and lungs of control and test mice. (a) ciliated and non-ciliated cells of normal trachea; (b) dying non-ciliated cell shows holes and missing microvilli after 12 weeks exposure to A-C; (c) normal cross-section of lung; (d) uneven raised areas in lung after 12 weeks exposure to carbon; (e) normal alveoli showing thin alveolar walls and small pores; (f) fused and thickened alveoli and enlarged pores in lungs exposed to carbon for 12 weeks.

Conventional histopathologic examination of tissues from the respiratory tract indicated the presence of carbon-loaded macrophages throughout the lung parenchyma in all samples exposed to carbon or A-C. No other changes were found, again demonstrating the greater sensitivity of SEM examination for detection of subtle changes in the respiratory epithelium.

## Hematology and Clinical Chemistry

Ten individually marked mice from air, carbon only, and A-C exposure groups were designated for repeated orbital bleeding after 0,1,4,8,12,16 and 20 weeks exposure (3 hr/day, 5 days/week). Hematological parameters were measured and individual sera were saved for assay of immunoglobulin concentrations. Additional mice were sacrificed after the same exposure intervals and blood chemistry assays were done on the individually collected sera.

Data for initial and 20 week values of selected hematological parameters are shown in Table 25. Clinical chemistry data are summarized in Table 26. Although the levels fluctuated during the exposure they did not vary significantly from normal values reported for mice. With our necessarily small sample size, the variation observed was too great to allow detection of trends in the experimental data.

## Immunoglobulin Quantitation

Among the five main classes of immunoglobulins IgM, IgG, IgA, IgD and IgE, the first three are involved in defense against infection. IgM is the first antibody to appear in the serum of an animal after stimulation with a protein antigen. Each molecule contains 10 antigen binding sites and is an efficient antibody in certain *in vitro* tests and in certain aspects of antimicrobial immunity. IgM makes up 5 to 10% of the total antibody protein in serum and is usually supplemented by IgG antibody early in the response to antigen stimulation. At least 80% of the serum antibodies directed against the antigen belong to the class IgG which has several subclasses. In addition, IgG is the only immunoglobulin to cross the placenta. IgA is found in the serum as well as in saliva, colostrum, tears, and nasal and bronchial secretions. Secretory IgA is the predominant immunoglobulin in external secretions which play an important role in the protection of mucous membranes and conjunctiva.

Serum immunoglobulin concentrations have been quantified to determine which of the immunoglobulin classes are affected by the exposure to pollutant. The major site of air pollutant action is the lungs, making the secretory immunoglobulins which protect the respiratory membrane a target of interest. Unfortunately, there are many technical difficulties involved in obtaining nasal and bronchial washings from mice. However, study of serum immunoglobulin levels in experimental animals was still of interest since changes in concentration of serum immunoglobulins in man have been reported in studies of several chronic lung disorders. Elevated levels of serum IgA with normal IgM and IgG concentrations were observed by Biegel and Krumholz(47) in adults with chronic obstructive pulmonary emphysema. The mean

TABLE 25. EFFECT OF LONG-TERM EXPOSURE TO CARBON AND ACID-CARBON MIXTURES ON SELECTED HEMATOLOGICAL PARAMETERS

| | Duration of Exposure (weeks) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | | | | 20 | | | | | |
| | Air | | Carbon | | A-C | | Air | | Carbon | | A-C | |
| Blood Parameter | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Hct (%) | 49.5 | 1.0 | 49.0 | 0.7 | 48.0 | 0.3 | 50.5 | 0.7 | 50.0 | 1.3 | 49.7 | 0.8 |
| Hb (gm) | 14.6 | 0.2 | 14.6 | 0.2 | 14.6 | 0.2 | 15.1 | 0.2 | 15.1 | 0.3 | 15.3 | 0.3 |
| RBC ($x10^6/cm^3$) | 5.18 | 0.15 | 4.99 | 0.21 | 3.94 | 0.17 | 5.21 | 0.25 | 4.99 | 0.17 | 5.02 | 0.10 |
| WBC ($x10^3/cm^3$) | 11.2 | 0.9 | 12.0 | 1.0 | 9.5 | 0.4 | 8.9 | 0.9 | 11.0 | 0.9 | 8.1 | 0.8 |
| Retic (% RBC) | 2.9 | 0.5 | 3.8 | 0.4 | 3.3 | 0.5 | 0.8 | 0.1 | 0.8 | 0.1 | 0.7 | 0.1 |
| Platelet ($x10^3/cm^3$) | 1030 | 48 | 1077 | 35 | 993 | 48 | 1097 | 52 | 1139 | 89 | 1388 | 131 |
| Diff - Neut | 16.3 | 2.3 | 21.0 | 4.0 | 23.5 | 2.0 | 24.5 | 2.1 | 23.0 | 2.4 | 31.4 | 2.9 |
| Lymph | 83.3 | 2.3 | 78.2 | 3.9 | 75.5 | 1.9 | 74.3 | 2.4 | 76.5 | 2.3 | 68.1 | 2.8 |
| Eosin | 0.3 | 0.3 | 0.8 | 0.3 | 0.9 | 0.5 | 0.4 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| Baso | 0 | 0 | 0 | 0 | 0 | 0 | 0.1 | 0.1 | 0 | 0 | 0 | 0 |
| Mono | 0 | 0 | 0 | 0 | 0.1 | 0.1 | 0.7 | 0.3 | 0.3 | 0.3 | 0.3 | 0.2 |

TABLE 26. EFFECT OF LONG-TERM EXPOSURE TO CARBON AND ACID-CARBON MIXTURES ON SELECTED CLINICAL CHEMISTRY PARAMETERS

| Clinical Chemistry Parameter | Duration of Exposure (weeks) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | | 20 | | | | | |
| | Baseline[a] | | Air | | Carbon | | A-C | |
| | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Alk P (IU/l) | 116 | 5 | 37 | 6 | 35 | 6 | 27 | 3 |
| ICDH (IU/l) | 29 | 2 | 18 | 2 | 22 | 2 | 21 | 2 |
| Total Prot (mg/dl) | 4.6 | 0.1 | 5.6 | 0.1 | 5.2 | 0.1 | 5.4 | 0.2 |
| HBDH (IU/l) | 315 | 12 | 200 | 10 | 253 | 17 | 272 | 25 |
| LDH-L (IU/l) | 383 | 20 | 238 | 9 | 317 | 21 | 317 | 21 |

[a] Animals were sacrificed to obtain sufficient sera for the assays. Only one group of animals was sacrificed before exposure began when no exposure differences existed.

IgA and IgG levels in the sera of patients with farmer's lung disease were found to be significantly higher than those of normal individuals (48). Similarly, occupational exposure of workers to 0.5-2.7 ppm $NO_2$ for 6 to 8 hours/day for up to 6 years resulted in elevated levels of serum IgA and IgM but decreased IgG concentrations in most cases (49).

In the present study, individual sera from 8 to 10 marked mice exposed to either air, carbon only, or A-C mixtures were collected at selected intervals and quantitated for immunoglobulin concentrations by radial immunodiffusion.

After 1 week exposure, serum levels of $IgG_1$ were significantly decreased in both the carbon and A-C groups compared to controls held at ambient conditions (Table 27). Levels of $IgG_{2b}$ and IgM were significantly increased compared to the controls. Serum IgA levels were not affected.

After 4 weeks exposure to A-C, serum levels of $IgG_1$, $IgG_{2a}$, and IgM were all significantly depressed compared to the controls. Again, IgA levels were not affected. Immunoglobulin levels in mice exposed to carbon only were not significantly different from the controls.

After 12 weeks, serum levels of $IgG_{2a}$ and IgM remained significantly depressed in mice exposed to A-C compared to air controls. The other immunoglobulin classes were not signficantly affected.

At the end of 20 weeks exposure to the pollutants, IgM levels continued to be suppressed in mice exposed to A-C. $IgG_{2b}$ was significantly increased in both A-C and carbon mice and IgA was reduced for the first time in mice exposed to both A-C and carbon, with the suppression being greatest in the mice exposed to carbon only.

In summary. concentrations of serum immunoglobulins can be measured and represent a sensitive parameter which is altered by exposure to low concentrations of carbon and A-C. In general, the changes observed were more marked in mice exposed to A-C than in those exposed to carbon alone. No changes were seen in serum levels of IgA until after 20 weeks of pollutant exposure when some depression was seen. An immediate depression in $IgG_1$ levels was seen which persisted in the A-C exposure group through 4 weeks of pollutant exposure. There was an initial slight increase in $Ig_{2a}$ levels at 1 week followed by depressed levels in A-C exposed mice through 12 weeks of pollutant exposure. Serum levels of $IgG_{2b}$ were significantly elevated after 1 week exposure to the pollutants (in contrast to depressed $IgG_1$ levels), were similar to controls during the intermediate intervals of exposure and were significantly elevated over the air controls at 20 weeks. IgM levels were initially increased at 1 week in both experimental groups after which they were significantly depressed only in the A-C exposed mice and remained so for the duration of exposure.

TABLE 27. LONG-TERM EXPOSURE TO ACID MISTS: IMMUNOGLOBULIN CONCENTRATIONS

| Exposure | | Concentration of Serum Immunoglobulin, mg/ml | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IgA | | IgG1 | | $IgG_{2a}$ | | IgG2b | | IgM | |
| Time, wk | Condition | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| 1 | Air | 0.19 | 0.01 | 1.46 | 0.11 | 1.56 | 0.28 | 0.54 | 0.04 | 0.15 | 0.02 |
| | Carbon | 0.20 | 0.02 | 0.98* | 0.15 | 1.81 | 0.24 | 0.83* | 0.17 | 0.23* | 0.03 |
| | A-C | 0.19 | 0.01 | 1.07* | 0.16 | 2.01 | 0.58 | 0.75* | 0.09 | 0.24* | 0.03 |
| 4 | Air | 0.23 | 0.01 | 1.47 | 0.16 | 2.77 | 0.21 | 0.74 | 0.11 | 0.22 | 0.02 |
| | Carbon | 0.21 | 0.02 | 1.59 | 0.33 | 2.54 | 0.28 | 1.00 | 0.15 | 0.17 | 0.02 |
| | A-C | 0.23 | 0.01 | 1.12* | 0.10 | 2.08* | 0.18 | 0.54 | 0.06 | 0.17* | 0.02 |
| 12 | Air | 0.44 | 0.07 | 2.24 | 0.31 | 2.70 | 0.26 | 0.72 | 0.09 | 0.25 | 0.02 |
| | Carbon | 0.40 | 0.04 | 2.28 | 0.26 | 2.77 | 0.31 | 0.89 | 0.14 | 0.20 | 0.02 |
| | A-C | 0.47 | 0.11 | 2.44 | 0.27 | 2.00* | 0.29 | 0.73 | 0.10 | 0.14* | 0.01 |
| 20 | Air | 1.07 | 0.11 | 1.69 | 0.21 | 2.85 | 0.33 | 0.56 | 0.10 | 0.25 | 0.03 |
| | Carbon | 0.75* | 0.10 | 2.56 | 0.47 | 3.27 | 0.60 | 1.34* | 0.39 | 0.18 | 0.03 |
| | A-C | 0.83 | 0.16 | 2.22 | 0.51 | 3.36 | 0.69 | 1.32* | 0.39 | 0.16* | 0.01 |

* Significant difference ($p \leq 0.05$) compared to air control.

Because a direct relationship between the levels of immunoglobulins and chronic pulmonary diseases is not established, it cannot be stated whether increases in concentrations of immunoglobulins reflect cause or effect. There have been no other results reported on the effects of exposure to sulfuric acid mists, with or without particulates, on serum immunoglobulin concentrations. Disruption of the elastin and collagen framework of the lung was reported for guinea pigs exposed to $NO_2$ (50). Such tissue damage could alter lung proteins so that they become antigenic and thereby stimulate the production of serum antibodies. An increase in titer of serum antibodies to normal lung proteins was observed in guinea pigs exposed to $NO_2$. Studies at IIT Research Institute (51) indicate that mice exposed to $NO_2$ for 12 weeks showed a marked decrease in concentrations (mg/ml) of serum IgA, and an increase in serum IgM, $IgG_1$ and $IgG_2$ immunoglobulins.

## Primary Antibody Response of Spleen Cells

The primary response to SRBC, as measured by the Jerne plaque assay, is the production of specific IgM antibody (52) and is a quantitative reflection of the ability of the animal to respond to an antigenic stimulus. In the Jerne plaque assay, lymphoid cells are incorporated together with a dense population of red blood cells in an agar layer. During incubation, each lymphoid cell that releases antibody causes the red cells near the lymphoid cell to become sensitized; the sensitized blood cells are then lysed, in the presence of complement, forming a "clear area" or plaque against a background of red cells. The effect on the primary response of spleen cells from mice exposed to the pollutants was determined after 4, 12, and 20 weeks of pollutant exposure. The SRBC were injected after the final pollutant exposure and the individual spleens were removed and tested four days later. The results (Table 28) are reported as plaque-forming units (PFU) per total spleen cell population.

TABLE 28. LONG-TERM EXPOSURE TO ACID MISTS: JERNE PLAQUE ASSAY FOR PRIMARY ANTIBODY RESPONSE

| Duration of exposure, wk | Plaque-forming cells/spleen x $10^4$ following exposure to: Air | | Carbon | | Acid-Carbon | |
|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE |
| 4 | 2.14 | 1.01 | 7.41* | 1.22 | 4.72 | 1.41 |
| 12 | 13.15 | 3.09 | 13.00 | 2.57 | 18.21 | 4.44 |
| 20 | 32.64 | 6.36 | 16.63* | 6.76 | 4.36*,a | 1.62 |

[*] Significant difference ($p < 0.05$) compared to air control.

[a] Significant difference ($p < 0.05$) compared to carbon exposure.

As expected, during the interval of exposure the number of PFU/spleen increased in normal (air-exposed) mice from $2.14 \times 10^4$ PFU/spleen at 4 weeks to $32.64 \times 10^4$ PFU/spleen after 20 weeks. After 4 weeks of pollutant exposure, an early stimulation of the response to SRBC was seen in mice exposed to either carbon alone or the A-C mixture. The increase in PFU/spleen in carbon-exposed mice was significant compared to the control mice, but not statistically different from the mice exposed to A-C. At 12 weeks after the beginning of pollutant exposure there were no differences between the experimental groups and controls. After 20 weeks of exposure there was a significant depression of PFU/spleen in mice exposed to carbon and even greater depression following exposure to A-C.

This appears to follow a classic pattern in which a short exposure to a stress causes an increased immune response and long-term exposure results in depression of that response. Thomas, Holt and Keast (53-57) have observed such biphasic changes in systemic spleen and regional lymph node responsiveness following exposure to tobacco smoke, with moderate exposure periods producing marked enhancement and prolonged exposure producing severe depression. Zarkower (58-59) noted a decrease in the overall ability of animals to form antibody after chronic exposures to carbon alone or in combination with $SO_2$. He observed an enhancement of antibody production in mediastinal lymph nodes after 102 and 135 days of exposure, but the effect was reversed by 195 days. A similar pattern was found in our studies with carbon and A-C exposures. Initially, there was an enhanced antibody response. After 20 weeks exposure, A-C exposure resulted in the greatest decrease in PFU/spleen, significantly less than both the control and carbon exposures.

Serum immunoglobulin levels and the plaque assay provide quantitative measurements of IgM production. In this study, the plaque assay measures response of spleen cells to antigenic stimulation while the other method records serum IgM levels in the absence of antigenic stimulation. There is some correlation between the results obtained by these two methods. It appears that the ability to respond to antigenic stimuli is not depressed as rapidly as background levels of serum IgM. The number of PFU/spleen was still increased after 4 weeks of exposure to pollutant when background IgM levels were depressed. The response of spleen cells to antigenic stimulation diminished from increased PFU/spleen after 4 weeks to marked depression after 20 weeks exposure. For both parameters, the more marked initial enhancement and subsequent depression was observed for the more severe stress of A-C exposure.

## Alveolar Macrophage

A limited number of experiments to examine the effect of A-C mixtures on the cellular defense system in the lungs of mice was conducted. Two approaches were taken: in the first, the mice were killed after pollutant exposure and alveolar macrophages lavaged from their lungs were examined for total cell counts and viabilities. In the second approach, the mice were infected with radioactively labeled *K. pneumoniae* by intranasal inoculation after the final pollutant exposure and bacterial inactivation was determined in the lungs.

After 4 weeks of exposure, viabilities and cell counts were done only on macrophages from one mouse from each group. The viability in the three exposure groups ranged from 93.5 to 95.5%. Total cell counts ranged from 5.7 x $10^5$ to 7.1 x $10^5$ cells. Table 29 shows the viability and total cell count values determined on alveolar macrophages lavaged from five to nine mice less than 1 hr after the last of a series of 12 or 20 weeks of exposure to carbon or A-C. There were no changes in viability at any of the exposure intervals. A trend toward decreased cell counts was observed in both the carbon and A-C groups after 12 weeks of exposure. No differences were observed after 20 weeks of pollutant exposure.

TABLE 29. LONG-TERM EXPOSURE TO ACID MISTS: VIABILITY AND TOTAL CELL COUNT OF ALVEOLAR MACROPHAGES LAVAGED FROM MICE

| Duration of exposure, wk | Number of mice/group | Viability, % | | | Total Cell Count x$10^5$ | | |
|---|---|---|---|---|---|---|---|
| | | Air | Carbon | A-C | Air | Carbon | A-C |
| 12 | 9 | 93.5 | 93.7 | 92.8 | 8.9 | 7.0 | 7.8 |
| 20 | 5 | 94.4 | 92.4 | 93.8 | 11.1 | 11.4 | 12.8 |

The effect of long-term carbon and A-C exposure on the bactericidal activity in the lungs of mice challenged with *K. pneumoniae* less than 1 hr after 4, 12, or 20 weeks of pollutant exposure is shown in Table 30. The number of mice used ranged from 5 to 21 per exposure group. The intranasally administered dose of *K. pneumoniae* was 2.5 x $10^5$ bacteria in 0.05 ml of 0.1% peptone water; the number of bacteria actually deposited in the lungs, as determined by radioactive counts, ranged from 1.9 x $10^3$ to 5.1 x $10^5$. The results show a significant increase in the percent of viable bacteria remaining, reflecting decrease in bactericidal activity, compared to the air controls in lungs of mice infected with *K. pneumoniae* immediately after 4 or 12 weeks exposure to either carbon alone or to A-C. At these exposure durations the percent bacteria remaining in the lungs was greater in mice exposed to A-C than to carbon alone, but not significantly so. The most significant factor in the decreased bactericidal activity in the lungs of these animals is apparently the loading of the macrophages with carbon. The slightly increased percent bacteria remaining in the lungs of mice in the experimental groups infected with *K. pneumoniae* immediately after 20 weeks of pollutant exposure was not statistically significant.

TABLE 30. LONG-TERM EXPOSURE TO ACID MISTS: BACTERICIDAL ACTIVITY IN LUNGS OF MICE

| Duration of exposure, wk | Bacteria Remaining (%) in Lungs of Mice Exposed to: Air |  | Carbon |  | A-C |  |
|---|---|---|---|---|---|---|
|  | Mean | SE | Mean | SE | Mean | SE |
| 4 | 40.1 | 2.9 | 53.8* | 3.0 | 55.3* | 4.7 |
| 12 | 15.5 | 2.5 | 24.0* | 1.9 | 29.7* | 2.3 |
| 20 | 51.0 | 4.7 | 58.1 | 6.2 | 53.4 | 5.1 |

* Significant difference ($p \leq 0.05$) compared to air control.

Infectious Challenge

After 4 and 20 weeks of exposure to the pollutant, mice were challenged within 1 hr of the final exposure with airborne influenza $A_2$/Taiwan virus. The lung lesions of surviving mice were scored 14 days later.

Although the infectious challenge dose was low, there was no enhancement of mortality in mice exposed for 4 weeks to either carbon alone or to the A-C mixture compared to air controls (Table 31). Pulmonary consolidation did not increase nor was there a decrease in mean survival time.

TABLE 31. LONG-TERM EXPOSURE TO ACID MISTS: RESPONSE OF MICE CHALLENGED WITH INFLUENZA $A_2$/TAIWAN VIRUS

| Duration of exposure, wk | Experimental Condition | Mortality D/T | Mortality % | RMSR (days) | Pulmonary Consolidation |
|---|---|---|---|---|---|
| 4 | Air | 16/164 | 10 | 13.5 | 2.05 |
|  | Carbon | 10/164 | 6 | 13.7 | 1.62 |
|  | A-C | 13/166 | 8 | 13.7 | 1.70 |
| 20 | Air | 48/133 | 37 | 12.2 | 3.11 |
|  | Carbon | 46/137 | 34 | 12.3 | 3.07 |
|  | A-C | 61/136 | 45 | 11.7* | 3.43* |

* Significant difference ($p < 0.10$) compared to both air control and carbon.

Increased mortality, markedly decreased ($p < 0.10$) mean survival time, and increased pulmonary consolidation compared to both the air controls to the mice exposed to carbon only was seen in mice exposed to A-C for 20 weeks. Hence, changes in the response to infectious challenge were detected following 20 weeks exposure to low concentrations of A-C mixtures.

Results from the 12-week exposure study suggest additional experiments which might yield information on the response of mice with a low-grade or latent infection to pollutant stress or to subsequent infectious challenge. Mice were exposed as in the other experiments. However, difficulties were encountered in controlling the relative humidity (RH) in the exposure chamber. Despite our efforts, which included increasing the dry air flow through the chamber and using trays of calcium chloride in the bottom of the chamber to lower the atmospheric RH to that which should be in equilibrium with a saturated solution of calcium chloride, the RH frequently reached 80 to 85% in the chamber by the end of a 3-hr exposure. This was undoubtedly an additional stress on the animals which appeared very ruffled and damp when they were removed after the exposure. This was especially noticed in the group exposed to carbon alone. Some of the mice in this group began to appear ill, and random deaths were recorded although they had received no infectious challenge. As a check of whether this simply represented the effect of exposure to pollutant or the activation of some latent infection, a few "sick" animals were sacrificed and the sera tested for antibody to various murine viruses. All three mice sacrificed had significant antibody titers against Sendai virus (1:10, 1:20, and 1:40). Latent infection with Sendai virus is known to cause problems in some mouse colonies and stressful conditions may activate the virus and kill the mice. Our control mice appeared healthy during these studies and thus no control mice were sacrificed for determination of murine antibody. Twenty-one animals died in the carbon group during the last 3 weeks of exposure while no deaths occurred in the A-C or air groups. The mice were challenged with airborne influenza virus after exposure to the pollutant for 12 weeks. The challenge dose was very high. However, the results are nonetheless of some interest.

| Experimental Condition | Mortality D/T | % | RMSR (days) | Pulmonary Consolidation |
|---|---|---|---|---|
| Air | 133/163 | 82 | 9.3 | 4.56 |
| Carbon | 144/148 | 97 | 7.8 | 4.95 |
| A-C | 147/162 | 90 | 8.7 | 4.81 |

Even with the high virus challenge, the increase in mortality and pulmonary consolidation in mice exposed to carbon or A-C was statistically significant. The increase in the carbon group (the "sick" group) is also significant over the A-C group. Apparently, the presence of a low grade or latent infection in animals exposed to the pollutant stress leaves them especially susceptible to a subsequent infectious challenge. Other studies have shown that conventional (not SPF) animals exhibit a lower tolerance for air contaminants than SPF animals (56).

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